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14. ABSTRACT
Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer. Because of the ease of synthesis and structural manipulation, small molecules with apoptosis-inducing ability have great potential to be developed into chemotherapeutic drugs. The b-lactam antibiotics have for the past 60 years played an essential role in treating bacterial infections without causing toxic side effects in the host. We hypothesized that active N-thiolated b-lactams can target a tumor-specific protein(s) and selectively induce apoptosis in human breast cancer but not normal cells. In this report, we have designed and synthesized a number of beta-lactams with selected C3 and C4 ring substituents, and evaluated their potencies to inhibit proliferation and induce apoptosis in human breast cancer, but not normal cells. We have also studied the biochemical targets of these b-lactams by synthesizing and using labeled compounds as well as by performing microarray assay. Our results supported by this IDEA award and the Concept Award strongly support our hypothesis that beta-lactams cause tumor DNA damage, which is responsible for their anti-tumor activities. Our studies have provided strong support for proof-of-concept of the potential use of these b-lactams in breast cancer prevention and treatment.

15. SUBJECT TERMS
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INTRODUCTION

Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer (1). Because of the ease of synthesis and structural manipulation, small molecules with apoptosis-inducing ability have great potential to be developed into chemotherapeutic drugs (2). One particularly important class of small molecule drugs, the beta-lactam antibiotics, have for the past 60 years played an essential role in treating bacterial infections without causing toxic side effects in the host (3, 4). Several years ago we uncovered new members of this family of drugs, termed N-thiolated beta-lactams, which are highly effective at inhibiting bacterial growth in drug-resistant strains of Staphylococcus aureus (5, 6). Their mode of action appears to differ from that of traditional beta-lactam antibiotics. Most innovatively, we have discovered and characterized, for the first time, the anti-proliferative and apoptosis-inducing properties of N-thiolated beta-lactam antibiotics against human tumor cells (7, 8). Based on these results, we hypothesized that active N-thiolated β-lactams can target a tumor-specific protein(s) and selectively induce apoptosis in human breast cancer but not normal cells. To test this innovative hypothesis, we have performed the proposed experiments as reported below.

BODY

For details, please see the included **APPENDICES**. Please also see below **KEY RESEARCH ACCOMPLISHMENTS**.

Novel N-thiolated beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells (8 and see Appendices) Historically, it has been shown that the beta-lactam antibiotics play an essential role in treating bacterial infections while demonstrating selectivity for prokaryotic cells. We recently reported that certain N-methylthio-substituted beta-lactam antibiotics had DNA-damaging and apoptosis-inducing activities in various tumor cells. However, whether these compounds affect human normal or nontransformed cells was unknown. In the current study, we first show that a lead compound (lactam 1) selectively induces apoptosis in human cancer, but not in the nontransformed, immortalized cells. Additionally, we screened a library of other N-methylthiolated beta-lactams to determine their structure-activity relationships (SARs), and found that lactam 12 has the highest apoptosis-inducing activity against human cancer cells, associated with increased DNA-damaging potency. Furthermore, we demonstrate that lactam 12, as well as lactam 1, potently inhibits colony formation of human prostate cancer cells. We also show that lactam 12 induces apoptosis in human ***breast***, prostate, and head-and-neck cancer cells. Finally, lactam 12 induces apoptosis selectively in transformed, but not in nontransformed and parental normal fibroblast, cells. Our results suggest that there is potential for developing this class of beta-lactams into novel anticancer agents.

Beta-lactams and their potential use as novel anticancer chemotherapeutic drugs (9 and see Appendices). The discovery of natural and synthetic antibiotics is one of the most important medical breakthroughs in human history. Many diseases, such as bacterial meningitis, pneumonia, and septicemia, are now curable with the use of antibiotics. Antibiotics are efficacious, generally well tolerated in patients, and have a low toxicity level. It is for these reasons antibiotics remain an attractive target for drug discovery. Traditional beta-lactam antibiotics (e.g. penicillins, penems, cephalosporins) have a bicyclic ring structure that is conformationally rigid and functions to inhibit bacterial cell wall synthesis. In addition to the bactericidal action of antibiotics, it has been discovered that many antibiotics are capable of inhibiting tumor cell growth. There are currently many antitumor antibiotics approved for cancer therapy, which work to inhibit tumor cell growth by DNA intercalation. The use of beta-lactams as prodrugs has also met with success by aiding delivery of the chemotherapeutic directly to tumor sites. Recently, a novel class of N-thiolated monobactams, so termed because they possess a monocyclic ring instead of the bicyclic ring, has been found to induce apoptosis potently and specifically in many tumor cell lines but not in normal, non-transformed cell lines. Other beta-lactams, such as the polyaromatics, have been found to slow or inhibit tumor cell growth, and the 4-alkylidene beta-lactams are capable of inhibiting matrix metalloproteinases and leukocyte elactase activity. These data indicate that synthesis and evaluation of beta-lactams are a promising area for further development in anticancer research.

Structure-activity relationships of N-methylthiolated beta-lactam antibiotics with C_3 substitutions and their selective induction of apoptosis in human cancer cells (10 and see Appendices). The development of novel anti-cancer drugs that induce apoptosis has long been a focus of drug discovery. Beta-lactam antibiotics have been used for over 60 years to fight bacterial infectious diseases with little or no side effects observed. Recently a new class of N-methylthiolated beta-lactams has been discovered that have potent activity against methicillin resistant Staphylococcus aureas. Most recently, we determined the potential effects of these N-thiolated beta-lactams on tumorigenic cell growth and found that they are apoptosis-inducers in human cancer cell lines. In the current study, we further determined the effects of the substitution of the *O*-methyl moiety on C_3 and stereochemistry of the beta-lactams on the anti-proliferative and apoptosis-inducing abilities. We have found that Lactam 18, in which C_3 is substituted with an acrylate ester group, is a very effective proliferation inhibitor against human premalignant and malignant ***breast***, leukemic, and simian virus 40-transformed fibroblast cells. Generally speaking, increasing the size of the moiety on C_3 decreases its anti-proliferation potency, possibly indicating steric hindrance with the cellular target or decreased permeability through the cell membrane. We also found that the stereochemistry of the beta-lactams plays an important role in their potency. The *3S,4R* isomers are more effective than their enantiomers (*3R,4S*), suggesting that *3S,4R* configuration is more favorable for target interaction.

This publication is directly related to **Task 1** outlined in the **Statement of Work**, “To evaluate requirements of the C_3 ring substituents of beta-lactams as a selective breast cancer cell apoptosis inducer”.

N-Thiolated beta-lactams: A new family of anti-Bacillus agents (11). This report describes the evaluation of N-thiolated beta-lactam antibiotics as potential anti-Bacillus agents. N-Thiolated beta-lactams are a new family of antibacterials that previously have been found to selectively inhibit the growth of Staphylococcus bacteria over many other genera of microbes. From the data presented herein, these lactams similarly inhibit a variety of Bacillus species, including Bacillus anthracis. The preliminary structure-activity studies suggest that there is a need to balance the lipophilic character of the C(3)/C(4) groups in order to obtain optimal anti-Bacillus activity. Elongation or extensive branching of the organothio substitutent diminishes antibacterial effects, with the sec-butylthio derivative providing the strongest activity.

N-thiolated beta-lactams: Studies on the mode of action and identification of a primary cellular target in Staphylococcus aureus (12). This study focuses on the mechanism of action of N-alkylthio beta-lactams, a new family of antibacterial compounds that show promising activity against Staphylococcus and Bacillus microbes. Previous investigations have determined that these compounds are highly selective towards these bacteria, and possess completely unprecedented structure-activity profiles for a beta-lactam antibiotic. Unlike penicillin, which inhibits cell wall crosslinking proteins and affords a broad spectrum of bacteriocidal activity, these N-thiolated lactams are bacteriostatic in their behavior and act through a different mechanistic mode. Our current findings indicate that the compounds react rapidly within the bacterial cell with coenzyme A (CoA) through *in vivo* transfer of the N-thio group to produce an alkyl-CoA mixed disulfide species, which then interferes with fatty acid biosynthesis. Our studies on coenzyme A disulfide reductase show that the CoA thiol-redox buffer is not perturbed by these compounds; however, the lactams appear to act as prodrugs. The experimental evidence that these beta-lactams inhibit fatty acid biosynthesis in bacteria, and the elucidation of coenzyme A as a primary cellular target, offers opportunities for the discovery of other small organic compounds that can be developed as therapeutics for MRSA and anthrax infections. ***Whether coenzyme A also acts as a primary target of beta-lactams in human breast cancer cells is being investigated.***

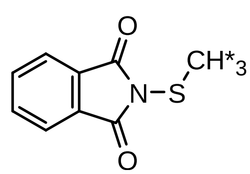
Preparation of radioactive β-lactams

1) Preparation of [H^3]-labeled *N*-Methylthio-Phthalimide (Scheme)

$$CH^*_3I + CH_3COSH \xrightarrow[CH_2Cl_2,\ 0^oC]{Et_3N} CH^*_3SCOCH_3$$

$$CH_3SSCH_3 \xrightarrow[-30^oC]{Cl_2} CH_3Cl \xrightarrow[-30^oC]{CH^*_3SAc} CH^*_3SSCH_3 + CH_3COCl$$

$$CH^*_3SSCH_3 + \text{Phthalimide} \xrightarrow[Br_2,\ 0^oC]{Pyr./MeCN}$$



Step 1: [H^3]-Radiolabeled Thioacetate

To a thio acetic acid 7.15 mL (0.1 mol) in methylene chloride (100 mL) at 0 ^{o}C under nitrogen atmosphere, diisopropylethyl amine 20 mL (0.11 mol) was added slowly over 10 min. The mixed idomethane 7.5 mL (0.12 mol) was added slowly over 30 min to the solution and stirred for 1 hr at 0 ^{o}C, and the reaction mixture was allowed to reach room temperature while stirring overnight.

Step 2: [H^3]-Radiolabeled Dimethyldisulfide

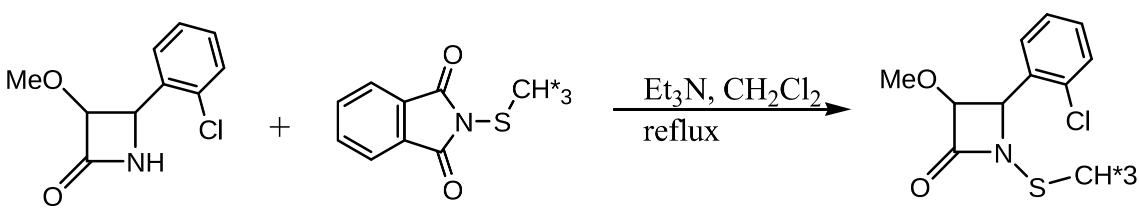
To a dimethyl disulfide 4.5 mL (50 mmol) at -30 ^{o}C, the chlorine gas 3.6 g (50 mmol) was added by bubbling. The methylthio acetate (38.3 mmol) solution prepared from above step 1 was added slowly to methylsulfenyl chloride over 30 min at -30 ^{o}C ~ -20 ^{o}C, and the reaction mixture was stirred for 1 hr at 0 ^{o}C. Upon completion, the resulting solution wa distilled immediately. Radiolabeled dimethyldisulfide 7.3 g was able to be obtained at 109 ^{o}C.

Step 3: [H^3]-Radiolabeled Methylthiophthalimide

The radiolabeled dimethyldisulfide 2.39 mL (26.5 mmol) prepared from step 2 and phthalimde 4 g (27 mmol) was dissolved in pyridine 20 mL and acetonitrile 25 mL solvent. To the solution at 0 ^{o}C, bromine 1.54 mL (30 mmol) in acetonitrile 30 mL was added slowly over 1 hr, and the reaction mixture was stirred for 1 hr at 0 ^{o}C and 1 hr at room temperature. Water 100 mL was added to the reaction mixture over 30 min at 0 ^{o}C and the solution was left stand for 1 hr. The precipitated crude product was filtered and washed with cold methanol to give 6.75 g (70 % yield) white solid.

2-(Methylthio)isoindoline-1,3-dione: white solid; ^{1}H NMR (250 MHz, CDCl3): mp 178-180^{o}C; δ 7.82 (m, 4H), 3.77 (s, 3H).

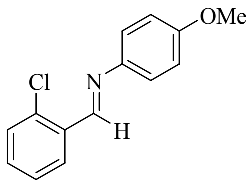
<u>2) Preparation of [H^3]-labeled *N*-Methylthio-β-lactam (Scheme)</u>



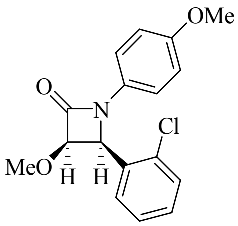
In a round bottom flask was added 0.1g (0.47 mmoles) *N*-protio β-lactam, 0.083g (0.47 mmoles) of radioactive *N*-methylthio-phthalimide and 0.087 mL (0.5 mmoles) of diidopropylethyl amine, in 5 mL of dichloromethane. The solution was refluxed and followed via TLC. Reaction was complete after 12 hours. After cooling, the solution was poured into an equal volume of water, washed with aqueous solutions of 5% sodium bicarbonate, 1% sodium bisulfate, and saturated sodium chloride. The extracts were then dried over magnesium sulfate and column chromatographed to give the radiolabeled β-lactam in 85% yield.

(±)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-*N*-(methylthio)azetidin-2-one: white crystal; mp 71-73 °C; ^{1}H NMR (250 MHz, CDCl3): δ 7.35 (d, 1H, *J* = 7.4 Hz), 7.24 (m, 3H), 5.29 (d, 1H, *J* = 4.9 Hz), 4.80 (d, 1H, *J* = 4.9 Hz), 3.16 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (63 MHz, CDCl3): δ 170.4, 133.8, 131.4, 129.6, 128.9, 126.8, 86.7, 62.7, 58.9, 21.8.

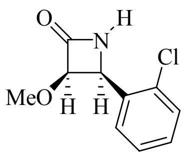
Preparation of *N*-protio β-lactam: The reactant, *N*-protio β-lactam, of the above reaction was prepared by general procedure through the following intermediates.



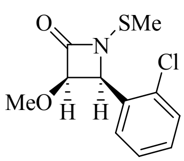
(E)-*N*-(2-Chlorobenzylidene)-4-methoxybenzenimine : yellow solid; mp 51-52 ^{o}C; ^{1}H NMR (250 MHz, CDCl, $CDCl_3$): δ 8.94 (s, 1H), 7.4-6.9 (m, 8H), 3.85 (s, 3H).



(±)-(3S,4R)-4-(2-chlorophenyl)-3-methoxy-1-(4-methoxy-phenyl)-azetidin-2-one : white solid; mp °C; IR (neat) 1747 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$, 250 MHz): re do proton; ^{13}C NMR ($CDCl_3$, 63 MHz): 163.6, 156.4, 133.2, 131.2, 130.3, 129.6, 128.9, 127, 118.6, 114.1, 84.9, 59.1, 58.8, 55.4.



(±)-(3S,4R)-4-(2-chlorophenyl)-3-methoxy-azetidin-2-one: white solid; mp 94-95 °C; IR (neat) 3275 cm^{-1} (N-H), 1770 cm^{-1} (C=O); ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.47 (d, 1H, *J* = 6.7 Hz), 7.39-7.27 (m, 3H), 6.97 (bs, 1H), 5.25 (d, 1H, *J* = 4.5 Hz), 4.83-4.82 (AB m, 1H), 3.26 (s, 3H); ^{13}C NMR (63 MHz, $CDCl_3$,) δ 168.5, 133.7, 133.1, 132.9, 129.2, 128.2, 126.9, 86.9, 59, 55.9.



(±)-(3S,4R)-4-(2-chlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one: white crystal; mp 71-73 °C; IR (neat) 1756 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.35 (d, 1H, *J* = 7.4 Hz), 7.24 (m, 3H), 5.29 (d, 1H, *J* = 4.9 Hz), 4.80 (d, 1H, *J* = 4.9 Hz), 3.16 (s, 3H), 2.40 (s, 3H); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 170.4, 133.8, 131.4, 129.6, 128.9, 126.8, 86.7, 62.7, 58.9, 21.8.

See References 13-18.

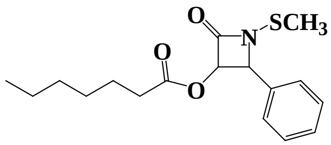
This section is directly related to **Task 1** outlined in the **Statement of Work**, "To evaluate requirements of the C_3 ring substituents of beta-lactams as a selective breast cancer cell apoptosis inducer".

To study the biochemical target of the labeled N-thiolated β-lactam. We have synthesized the [^{3}H-labeled]-beta-lactam 1 (see above). However, the radioactive label was very weak. We tried to use it to determine the binding proteins present in human breast cancer cells, but failed. We plan to repeat the labeling with modified conditions.

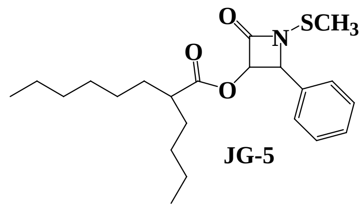
This section is directly related to **Task 1** outlined in the **Statement of Work**, "To evaluate requirements of the C_3 ring substituents of beta-lactams as a selective breast cancer cell apoptosis inducer".

<u>Unpublished Data: Effects of newly synthesized, novel β-lactams on growth of human breast cancer cells *in vitro* and *in vivo.*</u>

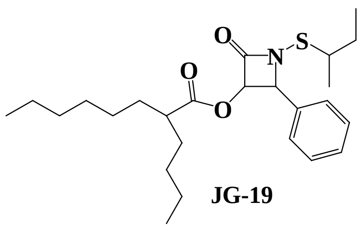
Organic Synthesis



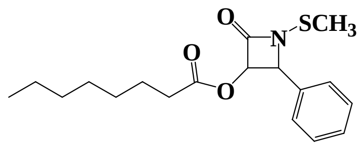
JG-3



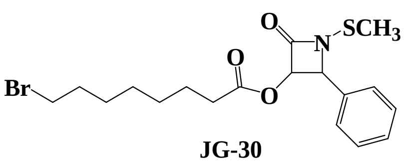
JG-5



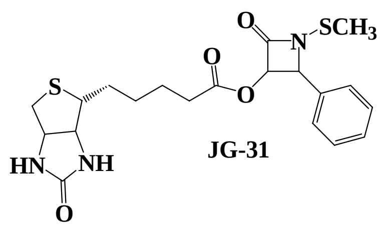
JG-19



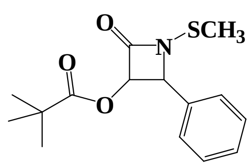
JG-20



JG-30



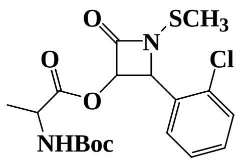
JG-31



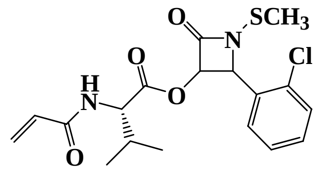
JG-32

Using the procedures outlined in the proposal, a variety of additional lactam analogues bearing oxygenated side chains at the C_3 center of the beta-lactam ring were synthesized. Our goal in building this library of compounds was to accommodate a diversity of structure types and polarities within the C_3 ester side chain in order to guage the effect of these side chains on *in vitro* anticancer activities. The first series included straight chain versus branched fatty ester derivatives JG-3 versus JG-5, the longer chain pairings of JG-19 versus JG-20, as well as haloalkyl ester JG-30 and biotinylated construct JG-31, and pivylate ester JG-32 (see above). These compounds supplement the long chain fatty ester derivatives that had been prepared in the prior funding cycle.

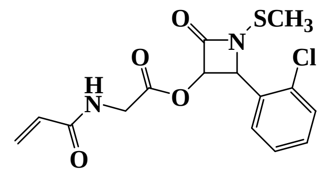
A second library of lactam derivatives was also constructed, this one comprised of more polar, and more highly water soluble, amino ester derivatives shown below. The selection includes N-BOC alanine (HY 14), N-acryloyl valine (HY 15), N-acryloyl glycine (HY 16), N-acryloyl proline (HY 18), and N-acryloyl methionine (HY 19). Two additional analogues, HY 20 and HY 21, having a succinyl linker unit between the lactam and amino ester side chain, were also prepared for evaluation.



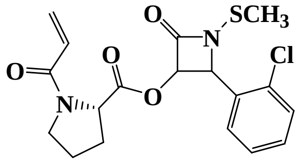
HY 14



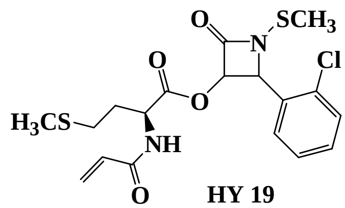
HY15



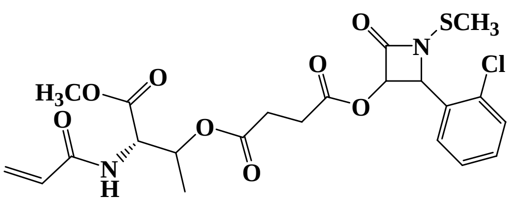
HY16



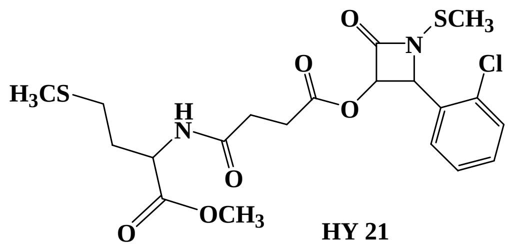
HY 18



HY 19

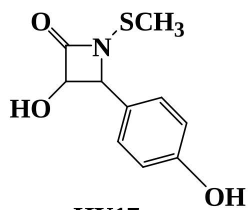


HY 20



HY 21

The un-acylated bis-hydroxyl lactam, HY 17, was also prepared for testing.



HY17

The compounds were all synthesized by acylation of the C_3 hydroxyl lactam with the appropriate amino acid using dicyclohexylcarbodiimide and N,N-dimethylaminopyridine in methylene chloride. The compounds were purified by flash chromatography on silica gel and their structures proven by proton and carbon NMR. Samples of each of the purified compounds were then sent to Professor Dou for anticancer screening.

Cell death-inducing activities of novel β-lactams. In order to discover more potent β-lactams against cancer, we have tested numerous of β-lactams that were synthesized by our chemistry collaborators. There are 8 compounds in HY group (HY14 to HY21) and 24 compounds in JG group (JG1 to JG 24). Chemical structures of some of the compounds are shown in Fig. 1. Our results indicate that in JG group, JG19 and JG5 were most potent cell death inducers when tested in human leukemia HL60 and Raji cell lines (Fig. 2A, B) and that in HY group, the order of potency to induce HL60 cell death was: HY20 > HY18 > HY16 = HY 15 > HY14 > HY19 > HY17 (Fig. 2C). Beta-lactam L-1 was used as a comparison (Fig. 2). The results from Western blot analysis also showed that JG19 and JG20 could induce PARP cleavage, a cellular apoptotic marker (Fig. 3).

Beta-lactams could effectively inhibit proliferation and induce apoptosis in human breast cancer cells. Previously we reported that L-1 has the great potency to induce apoptosis in cancer cells showed by MTT assay and PARP cleavage. In current experiment we screened more beta-lactams (Fig. 4) in order to discover more potent analogs. Human breast cancer MCF-7 cells were treated with each of the indicated beta-lactams at 1, 25 or 50 μM or DMSO (as solvent control) for 24 h, followed by performance of an MTT assay, which measures the status of cell viability and, thus, cell proliferation. After treatment with 50 μM of L-47, cellular viability of MCF-7 was decreased by 73%. Compared with 63% inhibition by 50 μM of L-1, L-47 was the most potent one in the tested beta-lactams (Fig. 5A).

We then treated another human breast cancer cell line MDA-MB-231 with 50 μM of L-1, L-30, L-47 or L-53 for different time points, followed by preparation of cell lysates and measurement of PARP cleavage, a cellular apoptotic marker, by Western blotting. The results showed that among the tested lactams, L-47 had the greatest potency to induce PARP cleavage within 8 h of treatment (Fig. 5B).

<u>This section is related to **Task 2** outlined in the **Statement of Work**.</u>

β-lactams could induce apoptotic cell death and increase Hsp70 protein expression. Previously we reported that L-1 (lactam 1) possesses apoptosis-inducing ability in transformed cells, but not non-transformed cells (7). Several β-lactams analogs, HY2, HY14 and HY15, were synthesized with substations at C_3 of the lactam ring (Fig. 6*A*) and assessed their cell killing effects compared to L-1. Human leukemia Jurkat T cells were treated with various concentrations of each of these β-lactams for 24 h, followed by a Trypan blue dye exclusion assay. The results showed that 25, 40, 43 and 50% cell death in Jurkat cells treated with 75 μmol/L of HY14, HY15, HY2 and L-1, respectively (Fig. 6*B*). In Western blot analysis, L-1 was also found to have the greatest potency to induce poly(ADP-ribose) polymerase (PARP) cleavage (Fig. 6*C*). Furthermore, we found that L-1 could dramatically increase the expression of heat shock protein 70 (Hsp70), which was a cellular stress related protein, in Jurkat cells (Fig. 6*D*).

Target genes of β-lactam L-1 in cancer cells. Based on results above, we chose L-1 as a lead compound to find the molecular target of β-lactams that induced DNA damage and apoptosis in cancer cells. Jurkat T cells were treated with 60 μmol/L of L-1 for 2 or 12 hours. Extracted RNA was converted to cDNA and analysed by the Affymetrix U133A chips and Affymetrix Microarray 5.0 (MAS 5.0) software. Over 22,000 genes were screened and their transcription levels at 2 and 12 hour treatment were analyzed. The results revealed a small number of genes that demonstrated an increasing more than two fold change at 2 hours treatment with L-1 compared to control (Table 1). A number of DNA interacting proteins were affected significantly such as GADD45, Inhibitor of DNA Binding 2 (ID2) and Zinc finger 38 (ZF38). Since these changes were consequently followed by the observation of DNA damage, we conclude that upregulation of these DNA-interacting proteins is likely an important component of cellular response. The largest transcriptional increase was observed in multiple isoforms of Hsp70 mRNA (isoform 1A increased by almost 67 fold at two hours). This dramatically upregulated gene transcription of Hsp70 was consistent with increased expression level of Hsp70 protein (Fig. 6*D*).

β-lactams were able to inhibit cell proliferation and induce apoptotic nuclear condensation in human breast cancer MDA-MB-231 cells. Our results revealed that L-1 had superior cell killing and apoptosis-inducing abilities in leukemia Jurkat T cells (Fig. 6). To verify whether the same effect of the β-lactams could be observed in solid tumor cell line, human breast cancer MDA-MB-231 cells were treated with various concentrations of the β-lactams for 24 hours, followed by a MTT assay. The results showed that the cell proliferation was inhibited by 27, 45, 52 and 62% in MDA-MB-231 cells treated with HY14, HY15, HY2 and L-1, respectively (Fig. 7*A*). The rank of cell killing and cell proliferation inhibitory ability was the same in both cell lines, which was, HY14 < HY15 < HY2 < L-1. To further confirm the apoptosis-inducing effect of the β-lactams, we measured apoptosis-associated nuclear morphological changes in MDA-MB-231 cells. After 24 hour treatment with 75 μmol/L of each β-lactam and Hoechst dye staining, L-1 generated more granular and brighter condensed nuclei compared with DMSO and other β-lactams treated cells (Fig. 7*B*).

β-lactams induce cell cycle arrest and DNA damage in MDA-MB-231 cells. To examine whether β-lactams were able to induce cell cycle arrest, MDA-MB-231 cells were treated with 50 μmol/L of tested β-lactams for 24 h, followed by propidium iodide (PI) and BrdU-FITC staining. According to cell cycle analysis by flow cytometry (Fig. 8*A*), L-1 increased the 34.3% of cells in the G_2/M phase, compared with 7.6, 8.5, 10.6 and 12.0% in DMSO, HY14, HY15 and HY2 treated cells, respectively. These results demonstrated that L-1 was able to induce cell cycle arrest with the greatest potency compared with other β-lactams. Furthermore L-1 increased the percentage of apoptotic sub-G_1 cells to 12.7% compared 0.8% of sub-G_1 cells treated with DMSO (Fig. 8*A*). BrdU-FITC labeling, often refered to as terminal deoxynucleotiddyltransferase dUTP nick end labeling (TUNEL), was used to detect the fragmented DNA which occurred during the apoptosis. After 24 h treatment of MDA-MB-231 cells with 50 μmol/L of L-1, 16.8% TUNEL-positive cells were observed compared with 0.47% in DMSO treated cells (Fig. 8*B*).

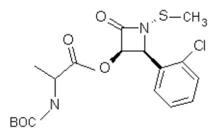
Beta-lactam HY14, an analog of L-47, significantly inhibits the growth of breast cancer xenografts, associated with its DNA damaging activity *in vivo* (*supported by the Concept W81XWH-04-1-0688, but not IDEA Award DAMD17-03-1-0175*).

*HY 14 was also used for animal testing and had to be made, pure, on a 50 mg scale. I would like to emphasize that this in vivo experiment has been approved by the ACURO office at the Office of Research Protections at the USAMRMC and the animal data shown in this report was supported by the Concept award (**W81XWH-04-1-0688**). I felt that it is necessary to mention the results in this report since these studies are related.*

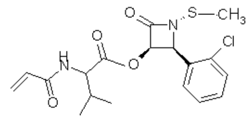
The data described above clearly demonstrate that β-lactams are apoptosis inducer in cultured leukemia and breast cancer cells. Our experimental results also showed that HY14 was more potent than L-1 in inducing cell death in cancer cells (Fig. 2C). Since we have a large quantity of HY14, we then examined anti-tumor activity of HY14 *in vivo*. We implanted MDA-MB-231 cells *s.c*. in nude mice. When the tumors became ~200 mm^3, the mice were *i.p*. treated with either vehicle control or HY14 at 0.3 or 3.0 mg/kg/day. The inhibition (up to 53%) of tumor growth by 3.0 mg/kg/day treatment of HY14 was observed after 30 days injection (Fig. 9A) but only 13% inhibition of tumor growth was showed by 0.3 mg/kg/day treatment, indicating that HY has anti-tumor activity which is dose-dependent (Fig. 9). The immunohistochemistry results showed that apoptosis-specific TUNEL positivity was found mainly in MDA-MB-231 tumors treated with HY14 at 3.0 mg/kg/day, less in those treated with 0.3 mg/kg/day of HY14, but none in vehicle-treated tumor (Fig. 9B). ***This result clearly demonstrates that beta-lactams cause tumor DNA damage in vivo, similar to what we reported in vitro*** (7, 8) and confirms that induction of DNA damage is responsible for their anti-tumor activities.

Development of these beta-lactams into potential antitumor drugs is our long-term goal. These studies should provide strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.

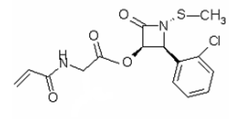
Figure 1. Chemical structure of related beta-lactams



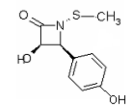
HY-14



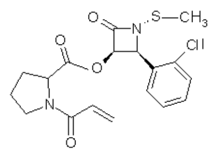
HY-15



HY-16



HY-17



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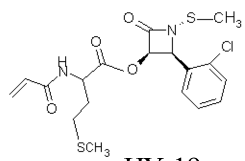
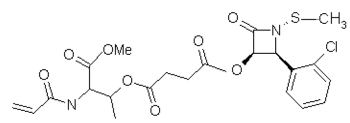
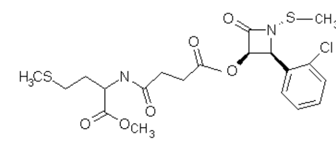


HY-19



HY-20



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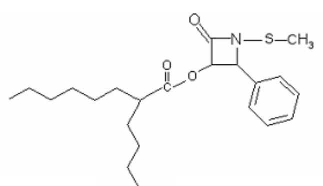
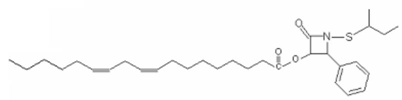


JG 5



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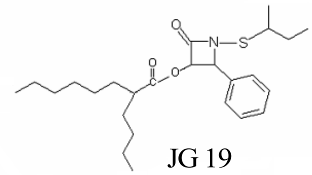

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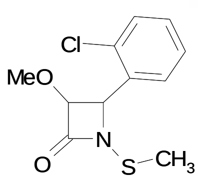
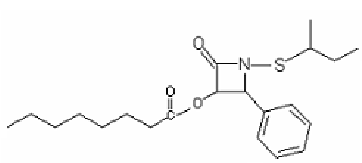


L-1



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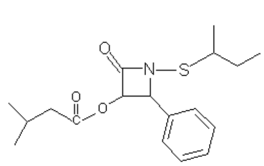
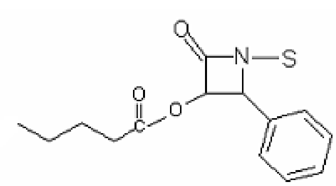


JG 22



JG 23

Figure 2. Beta-lactams could induce cell death in human leukemia cells.

Trypan blue assays: human leukemia HL60 (A, B) and Jurkat cell line (C) were treated with 50 or 100 μM of each beta-lactam for 24 hrs, and then non-viable cells were determined by Trypan Blue. L1 was used as a positive control.

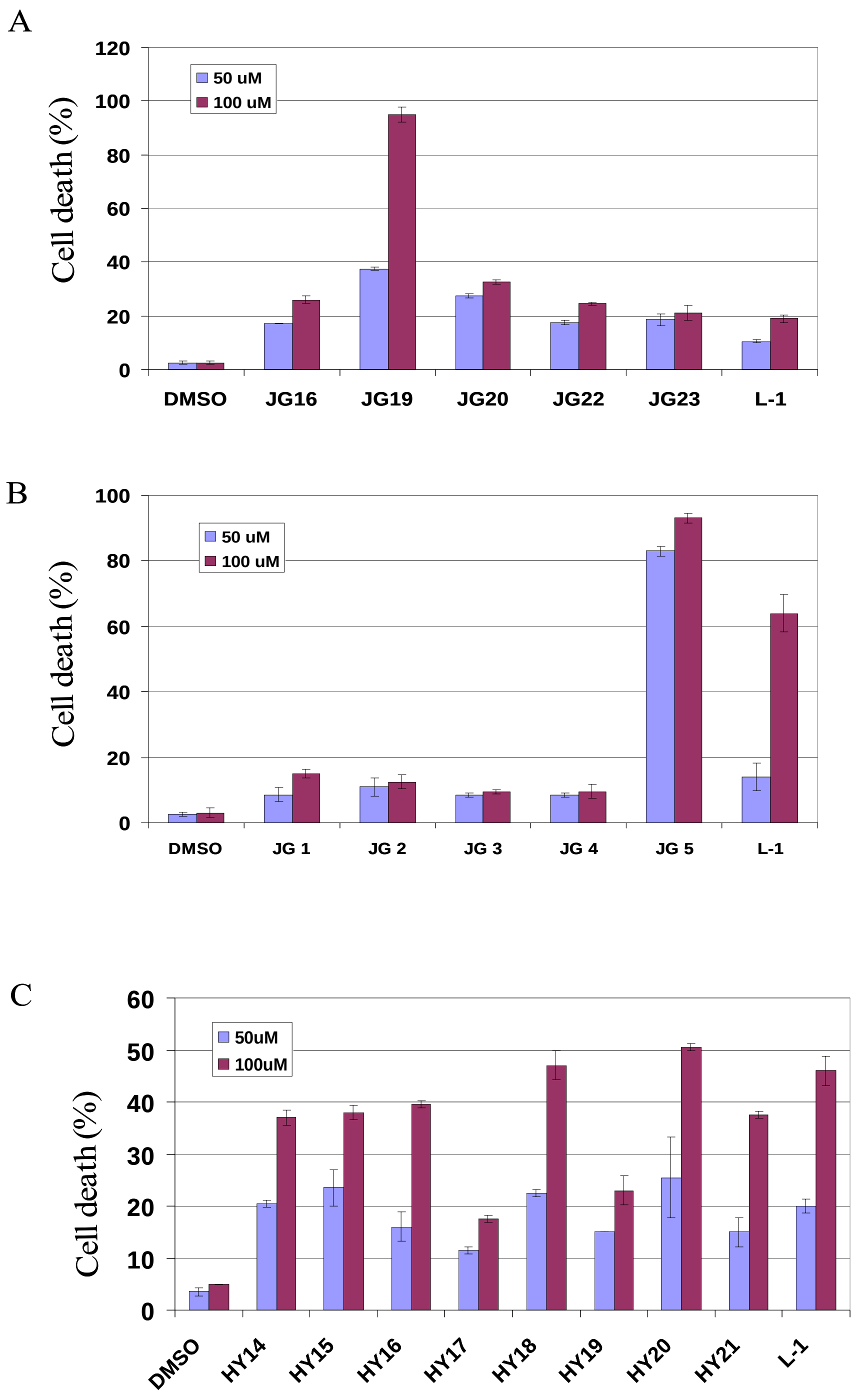


Figure 3. JG19 and JG20 could induce apoptosis-associated PARP cleavage.
Raji cells were treated with 50 uM JG19 or JG20 for 4, 8, or 20 hours before being harvested. The results show that both JG19 and JG20 induce PARP cleavage, indicative of apoptosis.

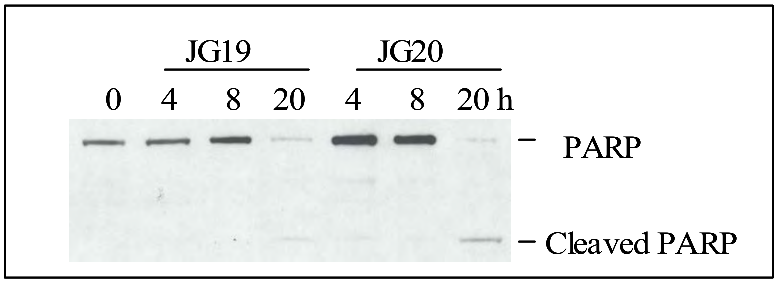
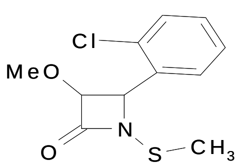
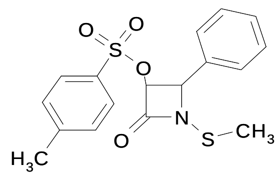
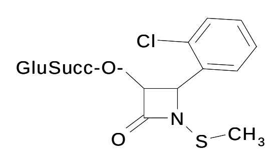


Figure 4. Chemical structures of more beta-lactams


Lactam 1


Lactam 30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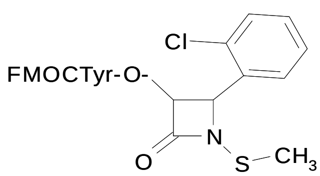
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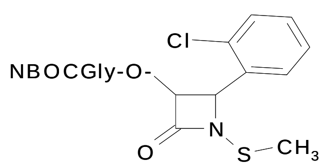
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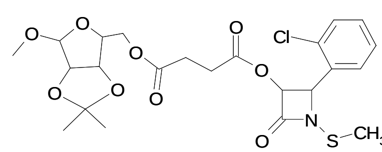
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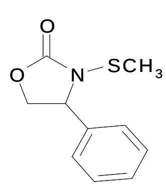
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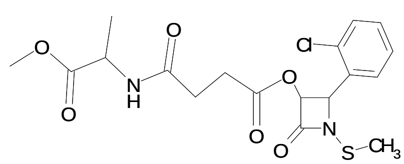
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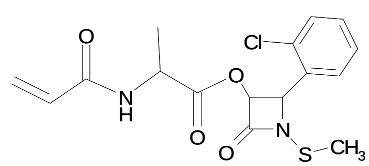
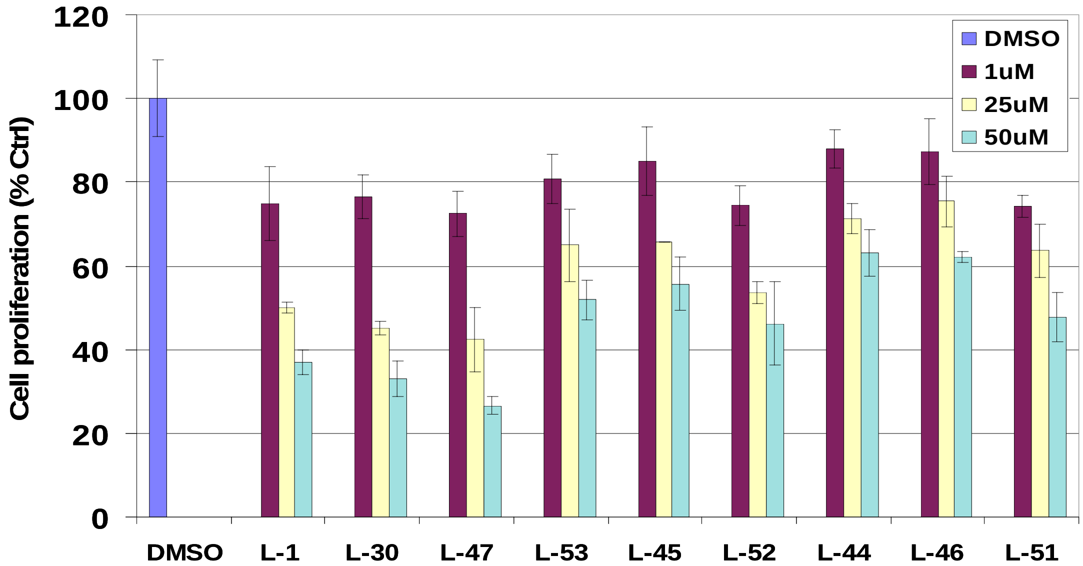

Lactam 53

Figure 5. β-lactams could effectively inhibit proliferation and induce apoptosis in human breast cancer cells.

A. MCF-7 cells were treated with either 1, 25 or 50 μM of indicated lactams or DMSO as solvent control (Ctrl) for 24 h, followed by performance of an MTT assay

B. MDA-MB-231 cells were treated with 50 μM of indicated b-lactams for different time points, followed by preparation of cell lysates and measurement of PARP cleavage by Western blot.

A



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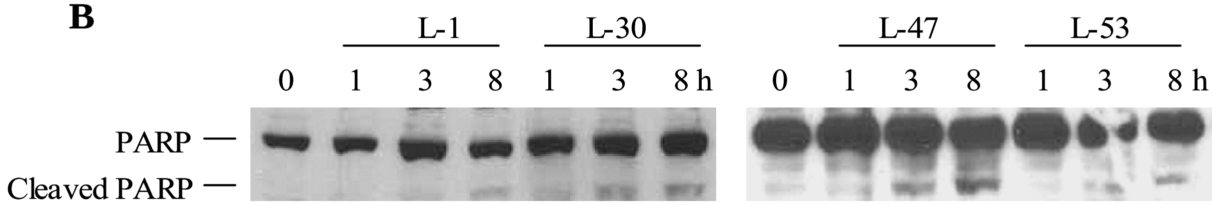


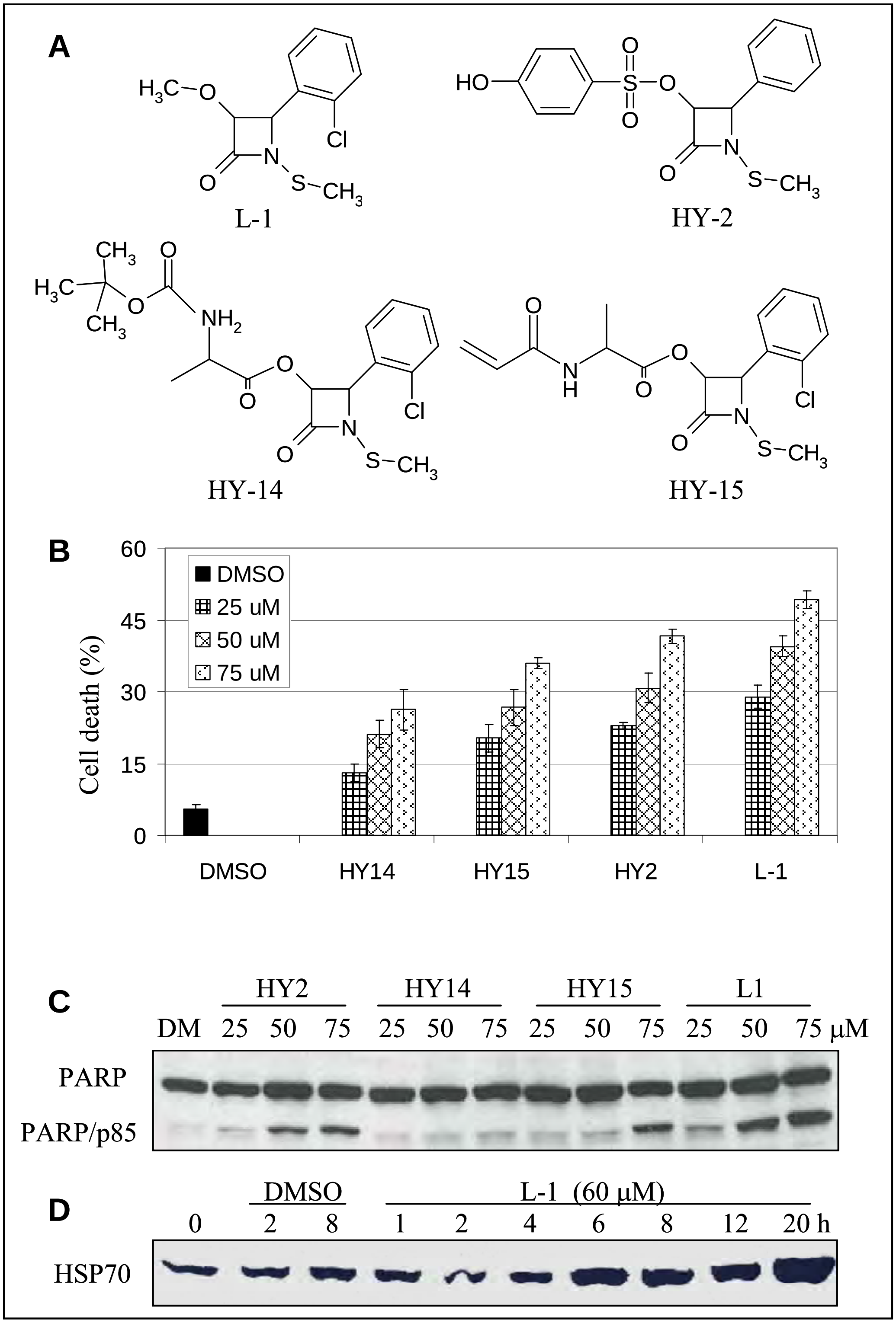


Figure 6. B-lactams could induce apoptotic cell death and increase Hsp70 protein expression in human leukemia Jurkat cells.

A. Chemical structures
B. Cell death in Jurkat cells measured by Trypan blue
C. PARP cleavage in Jurkat cell treated with indicated concentrations of lactams for 24 h
D. Expression of Hsp70 was increased in Jurkat cells treated with 60 µM of L-1.

Table 1. Genes with increased levels in Jurkat cells treated with L-1 for 2 or 12 hours.

Probe set ID number	Fold change 2h	12h	Accesion number and Gene name
207614_s	3.3	5.2	gb:NM_006352.1; Zinc finger 38
222074_at	3.3	4.1	gb:AW614435; Urophorinogen decarboxylase
201566_x	5.1	7.9	gb:D13891.1; Id-2H inhibitor of DNA binding 2
213988_s	6.5	17.1	gb:BE971383; Spermidinespermine N1-Acetyl Transferase
202912_at	3.2	11.9	gb:NM_001124.1; Adrenomedullin (ADM)
201565_s	5.3	18.9	gb:NM_002166.1; Inhibitor of DNA Binding 2 (ID2)
201739_at	7.1	20.1	gb:NM_005627; Serum Glucocorticoid Regulated Kinase (SGK)
202388_at	3.2	22.9	gb:NM_002923.1; Regulator of G-protein Signaling 2, 24KD (RGS2)
207574_s	9.2	56.7	gb:NM_015675.1; Growth Arrest and DNA Damage Inducible Beta (GADD45)
209304_x	43.7	254.2	gb:AF_087853.1; Growth Arrest and DNA Damage Inducible Beta (GADD45B)
202581_at	6.2	59.9	gb:NM_005346.2; HSP 70 Protein 1B
213418_at	7.8	410.2	gb:NM_002155.1; HSP 70 Protein 6
200800_s	66.9	3505.2	gb:NM_005346.2; HSP 70 Protein 1A

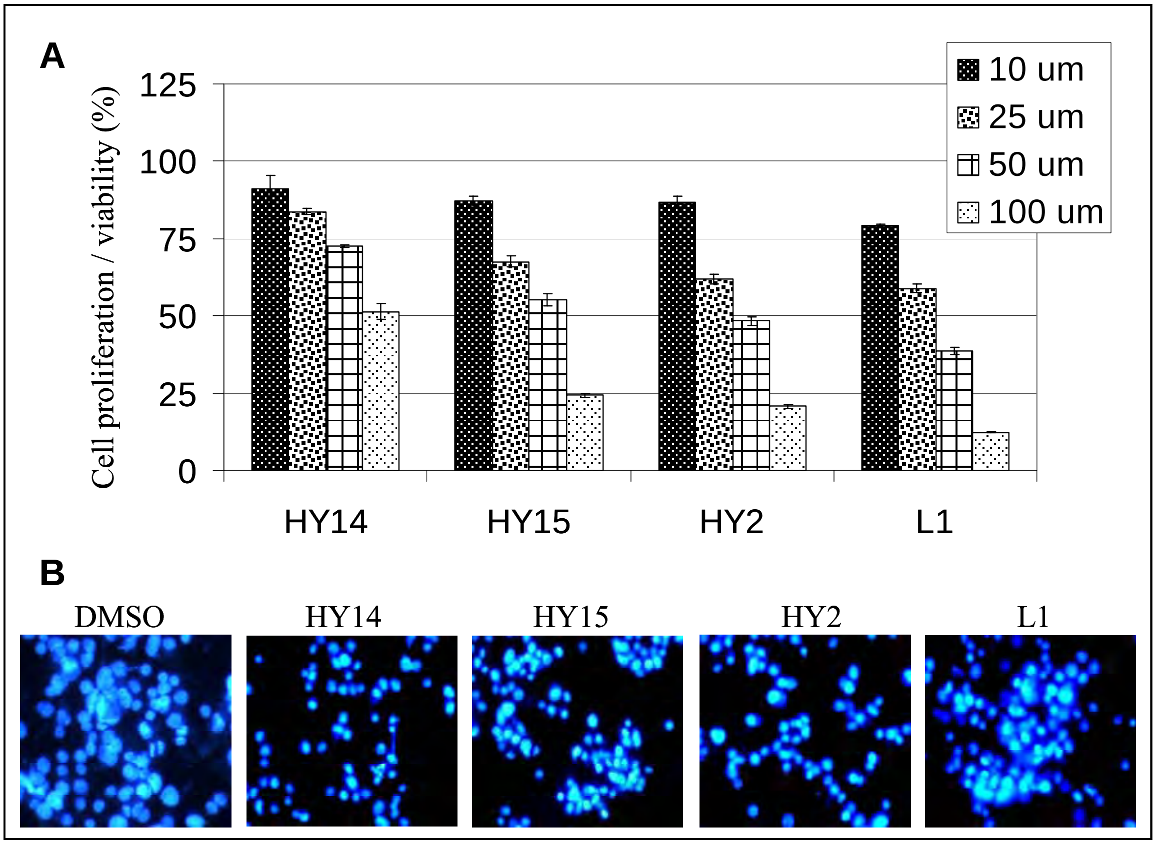
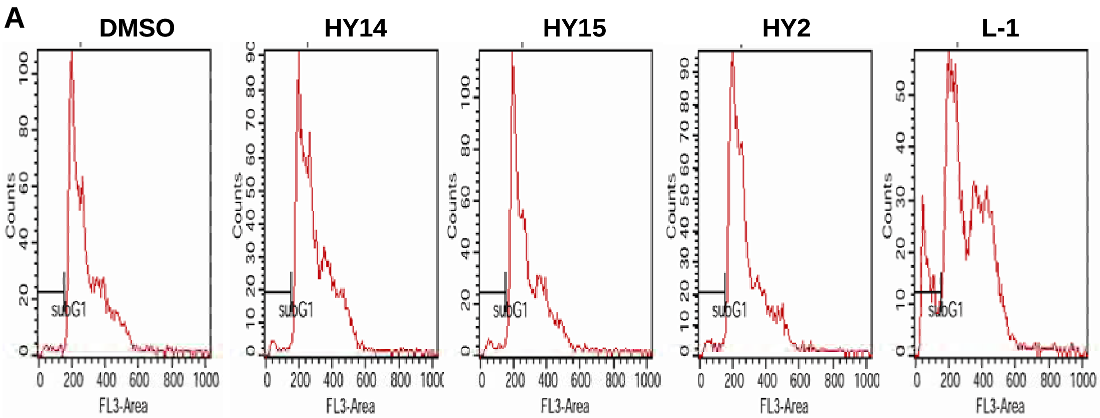


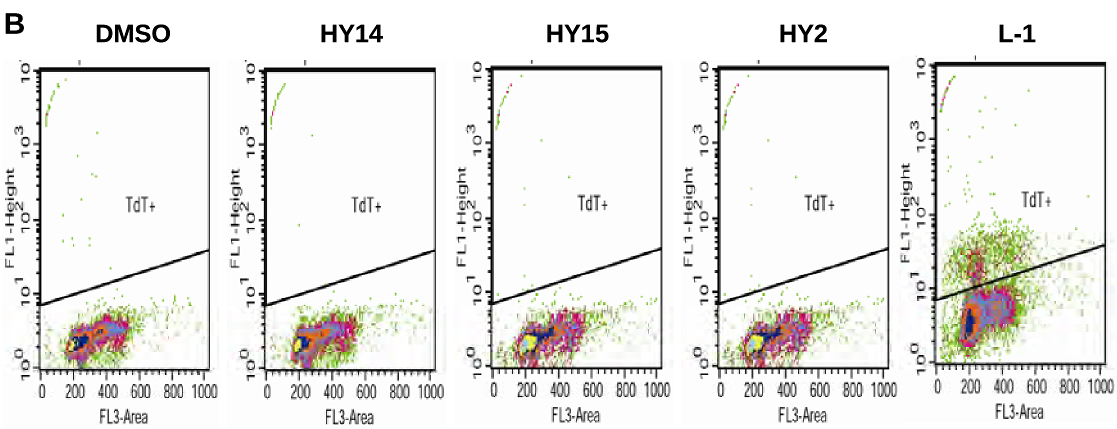
Figure 7. Lactams were able to inhibit cell proliferation in human breast cancer MDA-MB-231 cells and induce nuclear apoptotic changes

A. Cell proliferation was inhibited in MDA-MB-231 cells treated with lactams (MTT)

B. MDA-MB-231 cells were treated with 75 μM of β-lactams for 24 h, followed by Hoechst 33258 stain.



	DMSO	HY14	HY15	HY2	L-1
Sub-G1	0. 8	1. 6	1. 7	1. 0	12. 7
G0-G1	56. 5	51. 8	50. 3	54. 5	36. 5
S	35. 1	38. 1	37. 4	32. 5	16. 5
G2/M	7. 6	8. 5	10. 6	12. 0	34. 3



	DMSO	HY14	HY15	HY2	L-1
BrdU-FITC (%)	0.47	0.35	0.39	0.41	16.77

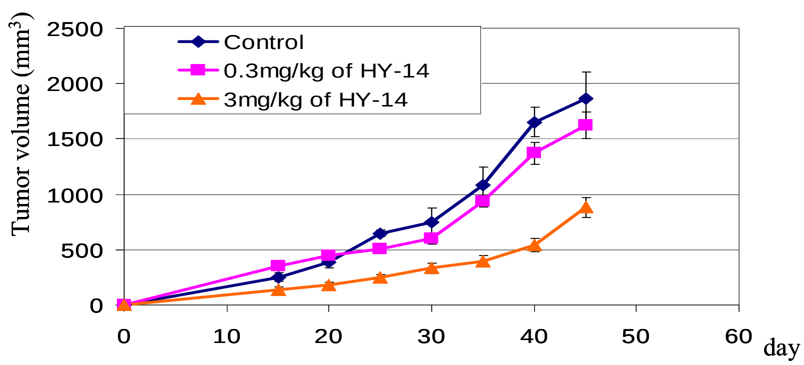
Figure 8. Among tested lactams, L-1 was most potent one to induce cell cycle arrest and DNA damage

MDA-MB-231 cells were treated with 50 μM of different lactams for 24h followed by Flow cytomitry measurement for cell cycle (A) and TUNEL (B).

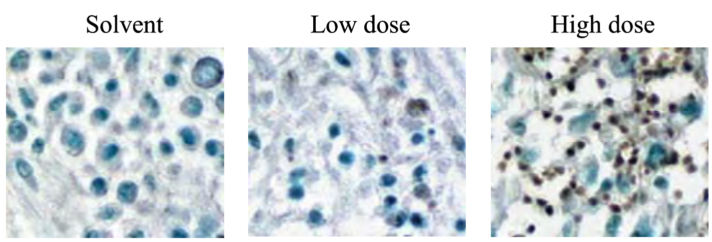
Figure 9. β-lactam HY-14 could inhibit the tumor growth in nude mice implanted by human breast cancer MDA-MB-231 cells.

Female athymic nude mice (NCRNU-M) were xenografted by injection of 6 X 10^6 MDA-MB-231 cells. 15 days after the injection, the mice were divided into three groups: solvent control, low dose (0.3 mg/kg) and high dose (3 mg/kg) treatment with β-lactam HY-14 by subcutaneous injection daily. Tumor size was measured every 5 days and tumor volume (V) was determined by the equation: $V=(L \times W^2) \times 0.5$, where L is the length and W is the width of a tumor. Tumor volume was calculated and expressed as cubic millimeters **(A).** TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) analysis of tumor tissues from three different groups was performed. Nuclei stained in dark brown indicate TUNEL positive **(B).**

A



B



KEY RESEARCH ACCOMPLISHMENTS

- Published 7 articles and 5 abstracts
- Gave 15 scientific presentations
- Trained three Ph.D. students
- Received a DOD Breast Cancer Research Program-Concept Award (**W81XWH-04-1-0688**) (PI: Q. Ping Dou).
- Received a Training Grant (**T32-CA09531-19 NIH**) (Ms. Kristin R. Landis-Piwowar; Mentor: Q. Ping Dou)
- NIH R01. N-Thiolated beta-Lactams. Co-Principal Investigator: Q. Ping Dou (PI: Ed Turos).
- Received a NIH R01 Award (**5 R01 CA120009**) (PI: Q. Ping Dou).
- Received a NIH R21 Award (Co-PI: Q. Ping Dou; PI: Jayanth Panyam).
- Partially supported several personnel (Deborah Kuhn, Ph.D., Kenyon Daniel, Ph.D., Di Chen, Ph.D., Huanjie Yang, Ph.D., Haiyan Pang, Ph.D., Shirley Orlu, B.S., Cindy Cui, B.S., Vesna Minic, M.S.)

REPORTABLE OUTCOMES

Provide a list of reportable outcomes that have resulted from this research to include:

Manuscripts (see Appendices):

Kazi A, Hill R, Long TE, Kuhn DJ, Turos E, Dou QP. Novel N-thiolated beta-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells. Biochem Pharmacol. 2004 Jan 15;67(2):365-74.

Kuhn D, Coates C, Daniel K, Chen D, Bhuiyan M, Kazi A, Turos E, Dou QP. Beta-lactams and their potential use as novel anticancer chemotherapeutics drugs. Front Biosci. 2004 Sep 1;9:2605-17.

Kuhn DJ, Wang Y, Minic V, Coates C, Reddy GS, Daniel KG, Shim JY, Chen D, Landis-Piwowar KR, Miller FR, Turos E, Dou QP. Structure-activity relationships of N-methylthiolated beta-lactam antibiotics with c3 substitutions and their selective induction of apoptosis in human cancer cells. Front Biosci. 2005 May 1;10:1183-90.

Daniel KG, Chen D, Orlu S, Cui QC, Miller FR, and Dou QP. Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells. Breast Cancer Res, 2005; 7:R897-R908

Landis-Piwowar KR, Chen D, Cui QC, Minic V, Becker FF, Banik BK, and Dou QP. Apoptotic-Inducing Activity of Novel Polycyclic Aromatic Compounds in Human Leukemic Cells. Intl. J Mol Med., 2006 May;17(5):931-5.

Edward Turos, Timothy E. Long, Bart Heldreth, J. Michelle Leslie, G. Suresh Kumar Reddy, Yang Wang, Cristina Coates, Monika Konaklieva, Sonja Dickey, Daniel V. Lim, Eduardo

Alonso and Javier Gonzalez. N-thiolated beta-lactams: a new family of anti-Bacillus agents. Bioorg Med Chem Lett. 2006 Apr 15;16(8):2084-90.

Kevin D. Revell, Bart Heldreth, Timothy E. Long, Seyoung Jang and Edward Turos. N-thiolated beta-lactams: Studies on the mode of action and identification of a primary cellular target in Staphylococcus aureus. Bioorg Med Chem. 2007 Mar 15;15(6):2453-67.

Abstracts (see Appendices):

Kazi A, Hill R, Long TE, Turos E, and Dou QP. Selective Induction of Apoptosis in Human Tumor Cells by Novel N-thiolated Beta-Lactams. Poster presentation. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of β-Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, Detroit, MI, September 8, 2004

Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping Dou, Ph.D. Relationships of structures of N-methylthiolated beta-lactam antibiotics to their apoptosis-inducing activity in human breast cancer cells. Poster presentation. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. β-Lactam antibiotics, Cancer Prevention and Treatment. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Landis-Piwowar KR, Wan SB, Daniel KG, Chen D, Chan TH, and Q. Ping Dou. Computational Modeling and Biological Evaluation of Methylated (-)-EGCG Analogs. Oral presentation. The 2006 AACR 97th Annual Meeting, Washington DC, April 1-5, 2006

Presentations:

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of β-Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, Detroit, MI, September 8, 2004

Dou QP. Invited Speaker. Synthetic beta-lactam antibiotics and a selective breast cancer cell apoptosis inducer: Significance in breast cancer prevention and treatment. The Breast Cancer Group, Karmanos Cancer Institute, Detroit, MI, May 6, 2004

Dou QP. Invited Speaker. Discovery of Novel Small Molecules: Rational Design, Structure-Activity Relationships, Cellular Targets, and Potential Uses for Cancer Treatment and Prevention. The Developmental Therapeutics Group, Karmanos Cancer Institute, Detroit, MI, September 8, 2004

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of β-Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

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Dou QP. Invited Speaker. Proteasome Inhibitors: Killing *via* Tumor-Specific Signaling. Basic and Translational Aspects of Cancer Cell Signaling Research Retreat, Karmanos Cancer Institute, Detroit, MI, January 14, 2005

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Dou QP. Invited Speaker. Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 24, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Therapies: - *From Nature to Laboratories and … back*. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 25, 2006

Dou QP. Invited Speaker. Discovery of Novel Natural and Synthetic Compounds for Molecular Prevention of Human Cancer. Henry Ford Health Systems, Detroit, MI, August 3, 2006.

Dou QP. Invited Speaker. Molecular Cancer Prevention and Therapies. Shandong Institute of Cancer Prevention and Treatment, Jinan, Shandong, China, October 18, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Prevention and Therapies. Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, China, October 20, 2006

Patents and licenses applied for and/or issued:
None

Degrees obtained that are supported by this award:

Kenyon Daniel, Ph.D., graduated from Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine on April 5, 2004. Dissertation Title: “Strategies for Cancer Therapy Through Regulation of Apoptotic Proteases” (Advisor: Q. Ping Dou). Currently working as a postdoctoral fellow at Moffitt Cancer Institute, University of South Florida.

Deborah Kuhn, Ph.D., graduated from Cancer Biology Program, University of South Florida College of Medicine on November 7, 2004. Dissertation Title: “Novel Approaches to Targeting Tumor Cell Apoptotic Signaling Pathways” (Advisor: Q. Ping Dou). Currently working as a postdoctoral fellow in University of North Carolina.

Kristin Landis-Piwowar, Cancer Biology Program, Wayne State University, **Scheduled to defend her Ph.D. degree on August, 2007**. Dissertation Title: “Synthetic Green Tea Catechin Analogs and Their Prodrugs: A Structural and Biological Analysis of (-)-Epigallocatechin-3-Gallate Variations” (Advisor: Q. Ping Dou).

Development of cell lines, tissue or serum repositories; infomatics such as databases and animal models, etc:
None.

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T32-CA09531-19 NIH Training Grant. “Training Program in the Biology of Cancer" (Ms. Kristin R. Landis-Piwowar; Mentor: Q. Ping Dou) 09/01/05-8/31/07.

NIH R01. N-Thiolated beta-Lactams. Co-Principal Investigator: Q. Ping Dou (5%) (PI: Ed Turos). 03/01/02-02/28/07. Total Direct Costs (to Dou lab): $200,000; Total Indirect Costs: $90,000

NIH R01. Roles of polymorphic COMT, tea polyphenols and proteasome in cancer prevention. 20% Effort (PI: Q. Ping Dou). 04/01/06-03/31/11. Total Direct Costs: $912,550; Total Indirect Costs: $460,842

NIH R21. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/07-06/30/09. Total Direct Costs: $250,000; Total Indirect Costs: $126,250 (2.8 percentile)

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

Deborah Kuhn, Ph.D.
Kenyon Daniel, Ph.D.
Huanjie Yang, Ph.D.
Haiyan Pang, Ph.D
Di Chen, Ph.D.
Shirley Orlu, B.S.
Cindy (Qiuzhi) Cui, Technician
Kristin Landis-Piwowar (Ph.D. defense on August, 2007)
Vesna Minic, M.S. (Ph.D. student)

CONCLUSIONS

We have designed and synthesized a number of beta-lactams with selected C_3 and C_4 ring substituents, and evaluated potencies of these synthetic beta-lactams to inhibit proliferation and induce apoptosis in human breast cancer cells. To study the biochemical target of these N-thiolated beta-lactams, we synthesized labeled compounds. However, the label was too weak to use. We have taken an alternative approach by performing a microarray assay and found a number of potential target genes regulated by beta-lactams, including GADD45 and Hsp70. We have also investigated and found these N-thiolated beta-lactams can induce apoptosis selectively in breast tumor *vs*. normal breast cells. Our results supported by this IDEA award and the Concept Award strongly support our hypothesis that beta-lactams cause tumor DNA damage, which is responsible for their anti-tumor activities. Development of these beta-lactams into potential antitumor drugs is our long-term goal. These studies should provide strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.

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APPENDICES

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Landis-Piwowar KR, Wan SB, Daniel KG, Chen D, Chan TH, and Q. Ping Dou. Computational Modeling and Biological Evaluation of Methylated (-)-EGCG Analogs. Poster presentation. The 2006 AACR 97th Annual Meeting, Washington DC, April 1-5, 2006

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Novel *N*-thiolated β-lactam antibiotics selectively induce apoptosis in human tumor and transformed, but not normal or nontransformed, cells

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Abstract

Historically, it has been shown that the β-lactam antibiotics play an essential role in treating bacterial infections while demonstrating selectivity for prokaryotic cells. We recently reported that certain *N*-methylthio-substituted β-lactam antibiotics had DNA-damaging and apoptosis-inducing activities in various tumor cells. However, whether these compounds affect human normal or nontransformed cells was unknown. In the current study, we first show that a lead compound (lactam **1**) selectively induces apoptosis in human leukemic Jurkat T, but not in the nontransformed, immortalized human natural killer (NK) cells. Additionally, we screened a library of other *N*-methylthiolated β-lactams to determine their structure–activity relationships (SARs), and found lactam **12** to have the highest apoptosis-inducing activity against human leukemic Jurkat T cells, associated with increased DNA-damaging potency. Furthermore, we demonstrate that lactam **12**, as well as lactam **1**, potently inhibits colony formation of human prostate cancer cells. We also show that lactam **12** induces apoptosis in human breast, prostate, and head-and-neck cancer cells. Finally, lactam **12** induces apoptosis selectively in Jurkat T and simian virus 40-transformed, but not in nontransformed NK and parental normal fibroblast, cells. Our results suggest that there is potential for developing this class of β-lactams into novel anticancer agents.

Keywords: *N*-thiolated β-lactam; Antibiotics; DNA damage; Apoptosis; Anticancer drugs

1. Introduction

Apoptosis, or programmed cell death, is a highly regulated process important in embryonic and immune system development and tissue homeostasis [1,2]. Perturbation of this pathway can lead to autoimmunity, acquired immune deficiency syndrome, neurodegenerative disorders, and cancer [3,4]. Initiation, commitment, and execution are the three fundamental steps of apoptosis [5]. Several apoptotic stimuli, such as death receptor-binding ligands, signal to activate the initiator caspases (caspases-2, -8, -9, -10), which in turn activates downstream effector caspases (caspases-3, -6, -7). The effector caspases can also be activated through the release of key mitochondrial proteins, such as cytochrome *c*, cell death inducer second mitochondria-derived activator of caspases (Smac), and apoptosis initiating factor [6]. It is generally believed that proteolytic cleavage of a variety of intracellular substrates, including poly(ADP-ribose) polymerase (PARP) [7,8] and the retinoblastoma protein (RB) [9–11], by effector caspases leads to apoptosis.

For nearly 60 years β-lactam compounds have been used in the treatment of bacterial infections [12]. Following the initial introduction of penicillin, a variety of other classes of β-lactam antibiotics were subsequently identified and used clinically, including cephalosporins, penems,

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Abbreviations: SAR, structure–activity relationship; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling; NK cells, natural killer cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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carbapenems, nocardicins, and monobactams [13]. The bacterial targets of these antibiotics are membrane-bound transpeptidases referred to as the penicillin-binding proteins, which are responsible for creating crosslinks within the bacterial cell wall [13]. By disrupting these cross-linking proteins, the β-lactams induce structural deformities within the cell wall, which cause the bacteria to lyse. Recently, a novel class of *N*-thiolated β-lactams has been shown to inhibit *Staphylococcus aureus* and methicillin-resistant *S. aureus* growth [14–16].

Previously we showed that *N*-thiolated β-lactams, such as β-lactam **1**, induced DNA damage, inhibited DNA replication, and induced tumor cell apoptosis in a time- and concentration-dependent manner [17]. Our current study shows, for the first time, that the *N*-thiolated β-lactam **1** can preferentially induce apoptosis in leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells. Additionally, we also show that lactam **12**, an analog of lactam **1**, has enhanced apoptosis-inducing activity in Jurkat T cells compared to lactam **1**. Furthermore, this study reveals that lactam **12** can induce apoptosis in other human solid tumor cell lines such as breast, prostate, and head and neck. Lactam **12** also induces apoptosis selectively in Jurkat T, but not human NK, cells, and in simian virus 40 (SV40)-transformed human fibroblasts (VA-13), but not in their parental counterpart (WI-38). Both lactams **1** and **12** are able to activate caspase-3 in human prostate cancer cells and inhibit colony formation of these cells in soft agar. These data indicate that further study of *N*-thiolated β-lactams in the treatment of cancer is warranted.

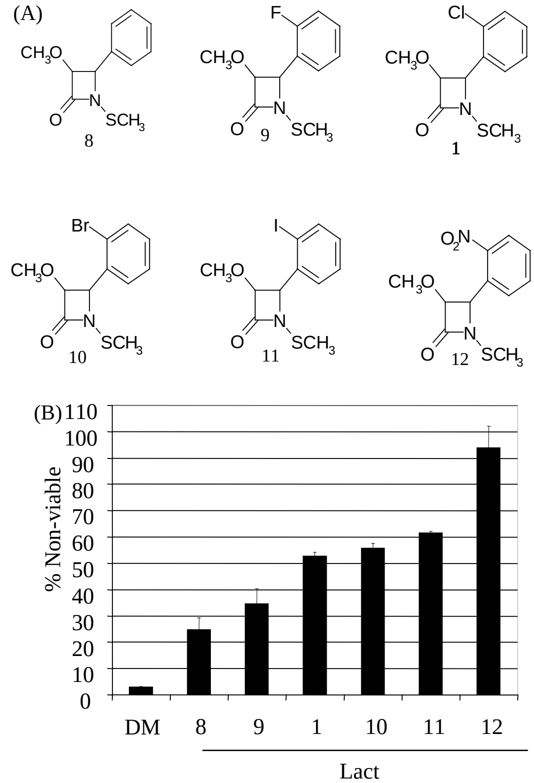

Fig. 1. Screen for more potent analogs of the lactam **1**. (A) Structures of the *N*-thiolated β-lactam compounds studied. Numerical designations were given to each compound. (B) Jurkat T cells were treated with the solvent (DMSO) or 50 μM of each indicated analog for 24 hr, followed by trypan blue dye exclusion assay. The numbers given are percentages of nonviable cells to total cells. Standard deviations are shown with error bars from a mean of at least three different experiments.

2. Materials and methods

2.1. Materials

Fetal bovine serum (Tissue Culture Biologicals), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue were purchased from Sigma-Aldrich. RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), MEM nonessential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen. Fluorogenic peptide substrate Ac-DEVD-AMC (the specific caspase-3/-7 substrate) was obtained from Calbiochem. Polyclonal antibody to human PARP was obtained from Roche Molecular Biochemicals. The APO-DIRECT kit for terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling (TUNEL) staining was purchased from BD Pharmingen.

2.2. Synthesis of β-lactams

The β-lactam analogs (Fig. 1A) were prepared as racemates (with *cis* stereochemistry) using a procedure described previously [14,15].

2.3. Cell culture, protein extraction, and Western blot assay

Human Jurkat T cells and human prostate cancer LNCaP cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human YT cells were cultured in RPMI 1640 medium supplemented with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM nonessential amino acids solution, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human breast cancer MCF-7 cells, head-and-neck cancer PCI-13 cells, prostate cancer DU-145 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in DMEM containing 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cell lines were maintained at 37° in a humidified incubator with an atmosphere of 5% CO_2.

A whole-cell extract was prepared as described previously [18]. Briefly, cells were harvested, washed with PBS and homogenized in a lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°. Afterwards, the lysates were centrifuged at 12,000 *g* for 15 min at 4° and the supernatants collected as whole-cell extracts. Equal amounts of protein extract (60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell) using a Semi-Dry Transfer System (Bio-Rad). The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to the proteins of interest.

2.4. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20 μL of cell suspension with 20 μL of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

2.5. Morphological assessment of apoptosis

To assess morphological changes of cells, 50 μL of treated or untreated cell suspension were transferred to a glass slide at the indicated time points. The slides were observed under a phase-contrast microscope (Leica) and photographs were taken (100×). Apoptotic cells were identified by their distinct morphological changes.

2.6. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was used to determine the extent of DNA strand breaks [19]. The assay was performed following manufacturer's instruction using the APO-Direct kit. In brief, the harvested cells were fixed in 1% paraformaldehyde for 15 min on ice, washed with PBS, and then fixed again in 70% ethanol at −20° overnight. The cells were then incubated in DNA labeling solution (containing terminal deoxynucleotidyl transferase (TdT) enzyme, fluorescein-conjugated dUTP and reaction buffer) for 90 min at 37°. After removing the DNA labeling solution by rinsing cells with Rinsing Buffer, the cells were incubated with the propidium iodide/RNase A solution, incubated for 30 min at room temperature in the dark, and then analyzed by flow cytometry within 3 hr of staining.

2.7. Caspase-3/-7 activity assay

To measure cell-free caspase-3/-7 activity, whole-cell extracts (20–30 μg) from untreated or treated LNCaP, MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were incubated with 20 μM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37° in 100 μL of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a VersaFluor™ Fluorometer (Bio-Rad) as described previously [20].

2.8. Soft agar assay

The soft agar assay was performed as described previously [21] with a few modifications. In brief, in a 6-well plate, a bottom feeder layer (0.6% agar) was prepared with DMEM media containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. A top layer (0.3% agar) was prepared with DMEM and the same media as described above but containing 2×10^4 prostate cancer LNCaP cells and 50 μM of lactam **1** or **12**, or equal volume of solvent (DMSO) as a control. Plates were incubated at 37° in 5% CO_2 in a humidified incubator for 3 weeks. MTT (1 mg/mL) was added to each well and incubated overnight to allow complete formation of purple formazan crystals. The plates were then scanned and photographed, and the number of colonies was quantified by *Quantity one* v. 4.0.3 software (Bio-Rad).

2.9. Nuclear staining

To assay nuclear morphology, the detached or remaining attached cells were washed with PBS, fixed with 70% ethanol for 1 hr, and stained with Hoechst 33342 (1 mM) for 30 min. The nuclear morphology was visualized by fluorescence microscopy (40×; Leitz) [18].

3. Results

3.1. Screening for more apoptotically active analogs of lactam *1*

Lactam **1** contains a *chloro* (–Cl) group in the *ortho* position on the benzene ring (Fig. 1A). To examine whether deletion or substitution of the Cl group could affect its cell death-inducing ability, other halogen and nonhalogen analogs of lactam **1** were synthesized (Fig. 1A). These compounds were then tested in the trypan blue dye exclusion assay, using lactam **1** as a comparison (Fig. 1B). Jurkat T cells were treated with each of these compounds at 50 μM for 24 hr, followed by measurement of loss of cell membrane permeability, indicative of a late apoptotic stage (Fig. 1B) [22,23]. As a control, lactam **1** induced ~52% cell death (Fig. 1B). Interestingly, removal of the Cl group from the benzene ring significantly decreased the cell death-inducing activity to ~25% (lactam **8**; Fig. 1B). Furthermore, replacement of the Cl group with a smaller halogen atom (–F; lactam **9**) also decreased the death-inducing activity (to ~35%), while analogs with a larger halogen group (–Br and –I; lactams **10** and **11**, respectively; Fig. 1A) increased the cell death rates to 55 and 60% (Fig. 1B). These data suggest that the size of the group in the *ortho* position on the benzene ring is important for the compound's cell death-inducing activity. Indeed, the analog with $–NO_2$ substitution, lactam **12** (Fig. 1A), exhibited the strongest effect with a total of

~94% cell death (Fig. 1B). Therefore, the order of potency of the tested compounds was: X = H < F < Cl < Br < I < NO_2.

3.2. *Lactam **1** induces apoptosis preferentially in leukemic Jurkat T over nontransformed, immortalized NK cells*

Previously, we reported that β-lactam analogs, such as lactam **1** (Fig. 1A) [17], were able to induce tumor cell apoptosis. However, whether lactam **1** affects normal or nontransformed cells was unknown. To determine whether lactam **1** was able to induce apoptosis preferentially in tumor/transformed vs. normal/nontransformed cells, we treated human leukemic Jurkat T cells and immortalized, nontransformed NK cells (YT line) [24] with lactam **1** in both concentration- and time-dependent experiments. Treatment of Jurkat T cells with 10 μM of lactam **1** for 24 hr induced apoptosis-specific PARP cleavage fragment p85 (Fig. 2A), whose levels were further increased when 25 μM of lactam **1** was used (Fig. 2A). After treatment with 50 μM of lactam **1**, PARP degradation was further increased, as evidenced by a significant decrease in expression of intact PARP protein (Fig. 2A). In contrast, no PARP cleavage was detectable in the YT cells after treatment with lactam **1** at even 50 μM (Fig. 2A).

In the kinetic experiment, both Jurkat T and YT cells were treated with 30 μM of lactam **1** for 3, 6, or 24 hr. PARP cleavage was detected in Jurkat T cells first at 3 hr, which was then increased at 6 hr (although the levels of PARP/p85 fragments at 24 hr were decreased in this Western blotting; Fig. 2B). Importantly, no PARP cleavage was observed in YT cells in the same kinetics experiment (Fig. 2B). To confirm the tumor cell-selective killing activity of lactam **1**, a trypan blue dye exclusion assay was performed in the same kinetic experiment. After 24 hr, there was 42% cell death in the Jurkat T cells compared to 9% in YT cells (Fig. 2C). Furthermore, by using phase-contrast microscopy, more cell death was observed in Jurkat T cells than YT cells (Fig. 2D). These data support the conclusion that lactam **1** could induce apoptotic cell death selectively in tumor *over* nontransformed cells.

3.3. *Lactam **12** has enhanced apoptosis-inducing activity specific to Jurkat T, but not normal YT cells*

To determine if lactam **12** is capable of inducing apoptosis at lower concentrations than lactam **1**, a dose–response experiment was performed with both compounds. Jurkat T cells were treated with lactam **12** at 2, 10, 25, and 50 μM for 24 hr, using 50 μM of lactam **1** as a comparison. Again, treatment with lactam **1** caused ~50% cell death, measured by trypan blue exclusion assay (Fig. 3A). Under the same experimental conditions, lactam **12** induced cell death in a concentration-dependent manner: 25% at 10 μM, 45% at 25 μM, and 80–90% at 50 μM (Fig. 3A). Therefore, lactam **12** is ~2-fold more potent than lactam **1**. This conclusion was further supported by PARP cleavage

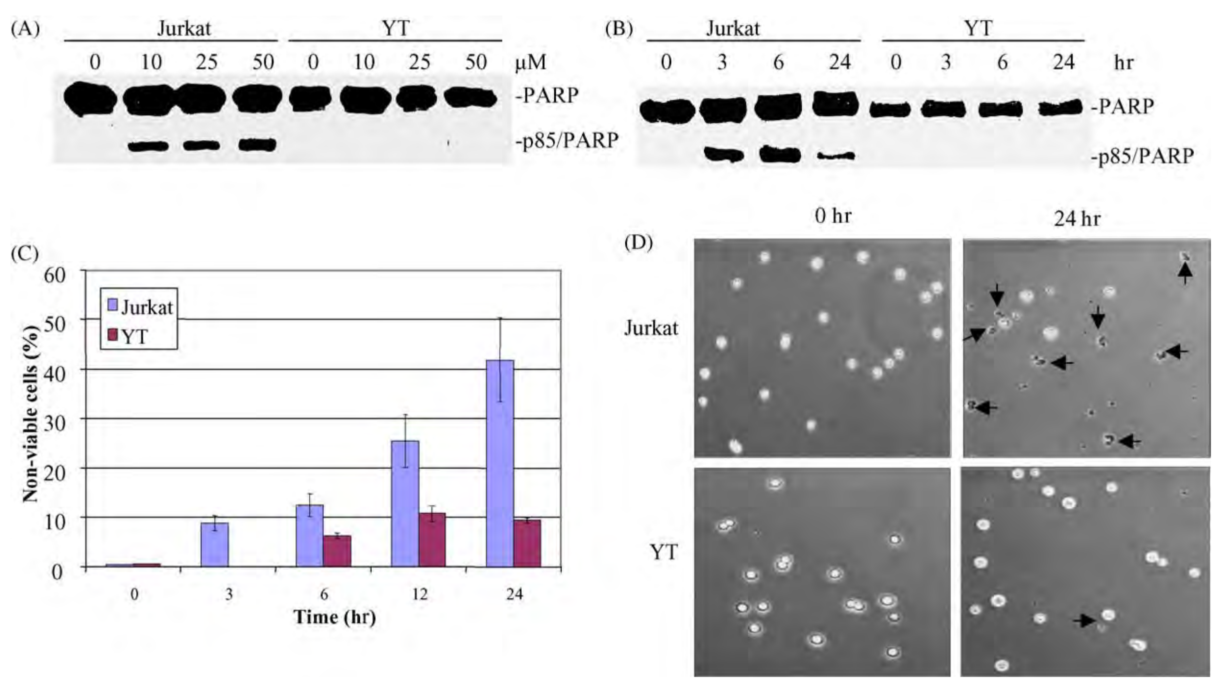

Fig. 2. Selective induction of apoptosis by lactam **1** in leukemic Jurkat T *over* immortalized/nontransformed NK cells. Jurkat T and NK (YT) cells were treated with 10, 25, and 50 μM of lactam **1** for 24 hr (A) or with 30 μM of lactam **1** for indicated hours (B–D). (A and B) Measurement of PARP cleavage in Western blot assay. The intact PARP (116 kDa) and a PARP cleavage fragment (p85) are shown. (C) Trypan blue dye exclusion assay. The numbers given are percentages of nonviable cells to total cells. Standard deviations are shown with error bars from a mean of at least three different experiments. (D) Morphological changes of Jurkat T and YT cells after treatment. Photographs under a phase-contrast microscope (100×).

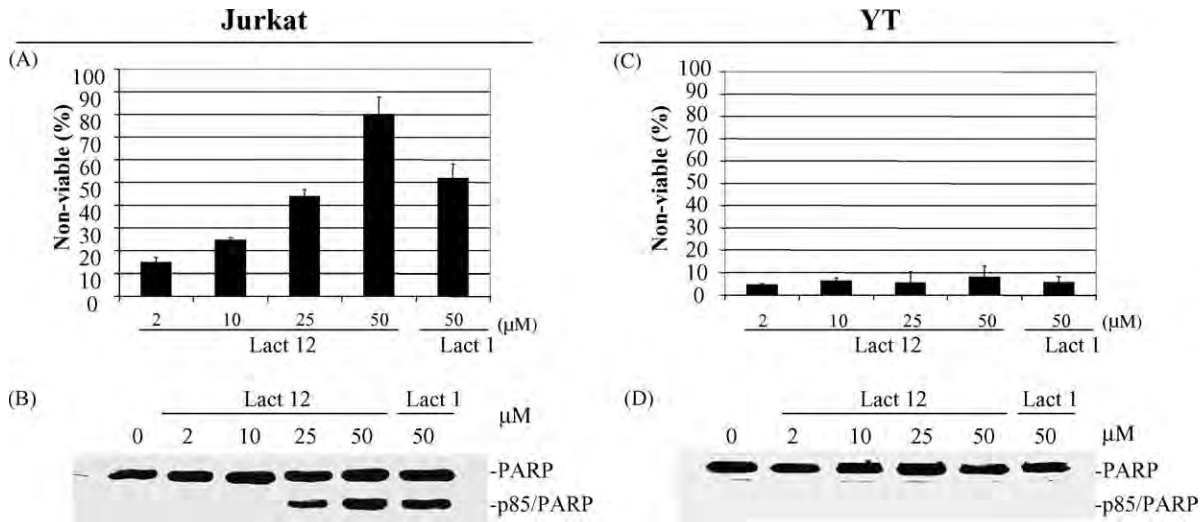

Fig. 3. Dose–response comparison between Jurkat T and YT cells treated with lactams **12** and **1** to induce cell apoptosis. Jurkat T (A and B) and YT cells (C and D) were treated with 2, 10, 25, and 50 μM of lactam **12** vs. 50 μM of lactam **1** for either 12 (B and D) or 24 hr (A and C), followed by trypan blue exclusion (A and C) or Western blot assay using anti-PARP antibody (B and D). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments (A and C).

assay using lysates prepared after 12-hr treatment (Fig. 3B). Cleavage of PARP occurred in lactam **12**-treated cells in a dose-dependent manner with the highest level of PARP cleavage observed at 50 μM (Fig. 3B). The levels of PARP cleavage induced by 50 μM of lactam **1** were equivalent to ~50% of that by 50 μM of lactam **12** (Fig. 3B).

In the same experiment, when immortalized, nontransformed NK cells were treated with lactam **12** (using lactam **1** as a control), neither cell death (Fig. 3C) nor PARP cleavage (Fig. 3D) were observed. Therefore, like lactam **1**, lactam **12** also induces apoptotic cell death preferentially in tumor *over* nontransformed cells.

To further compare the potency of lactams **1** and **12**, Jurkat T cells were treated with 25 μM of lactam **12** vs. 50 μM of lactam **1** for 3, 6, 12, and 24 hr, followed by determination of trypan blue incorporation and PARP cleavage. After 3 hr, lactam **12** at 25 μM caused 15% vs. 11% cell death with lactam **1** at 50 μM (Fig. 4A). Similarly, at 6 hr, 24% of trypan blue-positive cells were found after 25 μM lactam **12** treatment, while only 20% observed in 50 μM lactam **1**-treated cells (Fig. 4A). Only at later time points (12 and 24 hr), lactam **1** at 50 μM was slightly more potent than lactam **12** at 25 μM (Fig. 4A). Similar levels of cleaved PARP were observed in Jurkat T cells treated with either 25 μM of lactam **12** or 50 μM of lactam **1** at each time point (Fig. 4B). Therefore, lactam **12** is able to induce similar amounts of apoptosis in Jurkat T cells at a concentration half of that of lactam **1**.

Furthermore, we examined levels of sub-G_1 populations, as a measurement of cells with DNA fragmentation [18], in Jurkat T cells treated with lactam **12** or **1**. Treatment with 50 μM of lactam **12** increased the sub-G_1 populations by 34 and 57%, respectively, at 12 and 24 hr (Fig. 5A). In comparison, 50 μM of lactam **1** treatment for 12 and 24 hr induced sub-G_1 populations by 10 and 16%, respectively [17], confirming the greater potency of lactam **12**.

3.4. Lactam 12 is able to induce DNA damage in Jurkat T cells

We have previously shown that lactam **1** induces damage to DNA, leading to the inhibition of DNA replication and

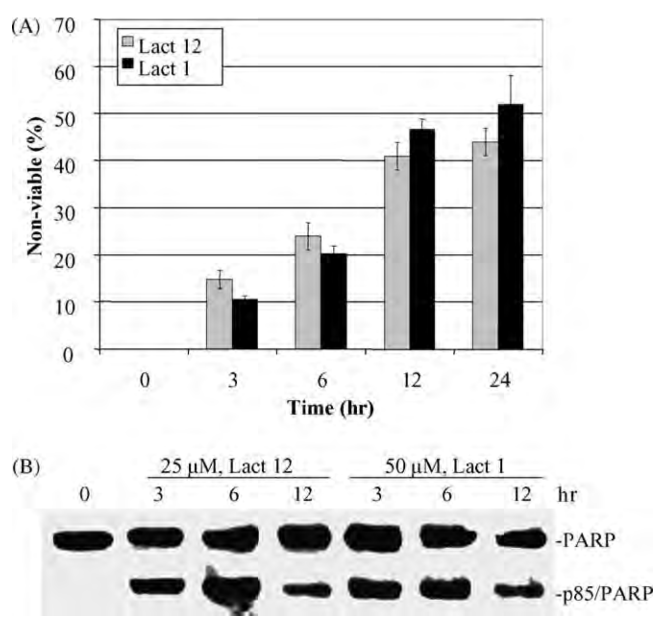

Fig. 4. A kinetic comparison between lactams **12** and **1** to induce apoptosis in Jurkat T cells. Jurkat T cells were treated with 25 μM of lactam **12** vs. 50 μM of lactam **1** for 3, 6, 12, and 24 hr, followed by trypan blue dye exclusion assay (A), and PARP cleavage in Western blot assay (B). Results are representative of three different experiments. Standard deviations are shown with error bars from a mean of at least three independent experiments.

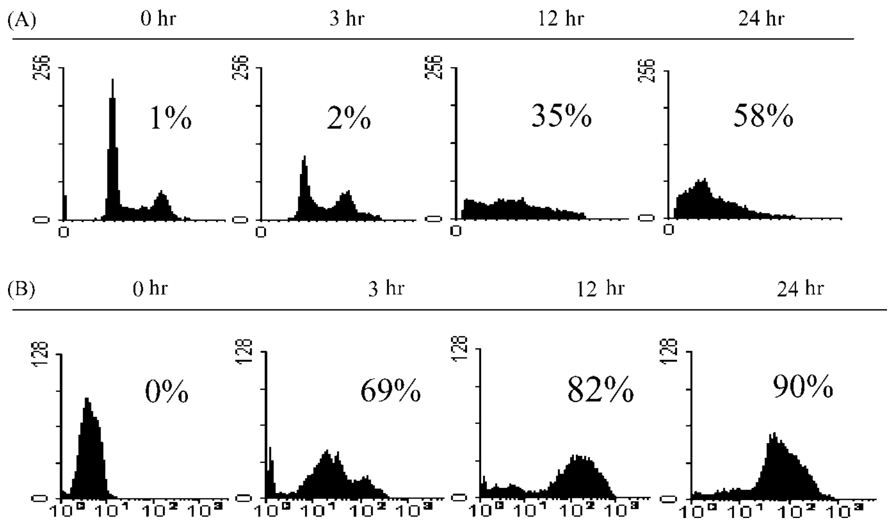

Fig. 5. Lactam **12** induces sub-G_1 cell population and TUNEL-positivity. Jurkat T cells (0 hr) were treated with 50 μM of lactam **12** for the indicated hours. (A) Measurement of sub-G_1 DNA content by flow cytometry analysis. The percentage of sub-G_1 cell population represents the cell populations with DNA fragmentation. (B) Measurement of DNA strand breaks by TUNEL assay. The numbers indicate the percentage of TUNEL-positive population. Results of representative of three independent experiments are shown.

subsequent induction of apoptosis [17]. To determine whether lactam **12** is also capable of damaging tumor cell DNA, Jurkat T cells were treated with 50 μM of lactam **12**, followed by performance of TUNEL assay, which detects DNA strand breaks [17]. A significant population (∼70%) of the cells exhibited DNA strand breaks after 3 hr of incubation with lactam **12** (Fig. 5B). A total of 82–90% of the cells were TUNEL-positive after 12–24-hr treatment with lactam **12** (Fig. 5B). In this experiment, 66% of TUNEL-positive cells were observed after treatment with 50 μM of lactam **1** for 24 hr (data not shown). Thus, the increased DNA-damaging capability of lactam **12** is most likely responsible for its enhanced cell death-inducing activity (Figs. 1–4).

*3.5. Lactams **1** and **12** induce apoptosis and inhibit colony formation in human prostate cancer cells*

So far, we demonstrated that lactam **12**, like lactam **1**, is able to induce DNA damage and subsequently induce apoptosis in human leukemia cells (Figs. 1–5) [17]. To determine if this lactam could also activate death program in solid tumor cells, human prostate cancer LNCaP cells were treated for 48 hr with lactam **12** at 2–25 μM or lactam **1** at 50 μM (as a control), followed by measurement of cell-free caspase-3/-7 activity. A dose-dependent increase in caspase-3/-7 induction was observed in LNCaP cells treated with lactam **12**: by 2-, 2.5-, and 4.2-fold, respectively, at 2, 10, and 25 μM (Fig. 6A). Treatment with 50 μM of lactam **1** also increased levels of caspase-3/-7 activity by 2.5-fold over the control (Fig. 6A). These data are consistent with the conclusion that lactam **12** has greater apoptosis-inducing potency than lactam **1**.

We then investigated the *in vivo* effects of these two lactams in a soft agar assay that measures the transforming activity of human tumor cells. LNCaP cells were plated in soft agar along with 50 μM of lactam **1**, 50 μM of lactam **12**, or solvent (DMSO), followed by a 21-day incubation to allow for colony formation. The solvent (DMSO)-treated plates allowed for the development of ∼500 colonies (Fig. 6B and C). Lactam **1** inhibited 91%, and lactam **12** completely blocked (∼100%), colony formation of LNCaP cells (Fig. 6B and C). Therefore, both lactams are able to inhibit the transformation capability of prostate cancer cells.

*3.6. Lactam **12** induces apoptosis in several solid tumor cell lines and SV-40-transformed, but not normal, human fibroblasts*

In a previous study, we showed that lactam **1** induced apoptosis in several solid tumor cell lines [17]. In this study we also investigated the effects of lactam **12** on several solid tumor cell lines including human breast (MCF-7), head-and-neck (PCI-13), and prostate (DU-145) cancer cells. Furthermore, we wanted to investigate whether lactam **12**-induced cell death was selective in transformed (VA-13) *over* the normal (WI-38) human fibroblasts. We treated these cell lines with 50 μM lactam **12** or an equal percentage of DMSO, followed by separation of the attached and detached cell populations. Both attached and detached cell populations were then used for detection of apoptotic nuclear change. We found that after a 48-hr treatment with lactam **12**, ∼60% of MCF-7 and PCI-13 cells and ∼50% of DU-145 and VA-13 cells became detached. However, no detachment was observed in WI-38 cells after treatment with lactam **12**. Little or no detachment was observed in all the cell lines treated with DMSO. All the detached tumor or transformed cells exhibited typical apoptotic nuclear condensation

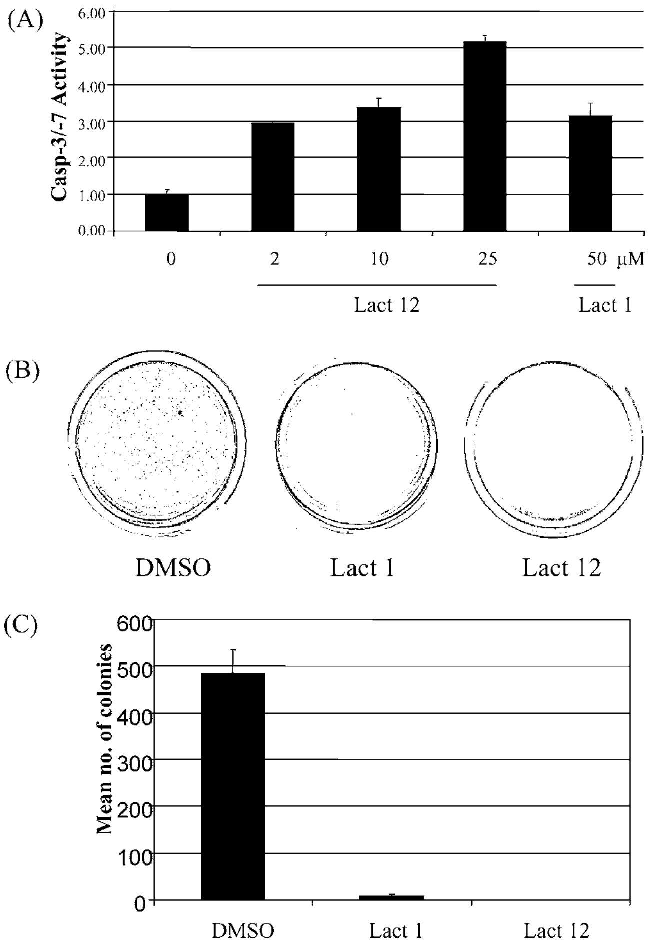

Fig. 6. Effects of β-lactams on caspase activation and colony formation. (A) Prostate cancer LNCaP cells were treated for 48 hr with 2, 10, and 25 μM of lactam **12** vs. 50 μM of lactam **1**. Cell-free caspase-3/-7 activity was then determined by incubating whole-cell extracts with caspase-3/-7 substrate and measuring free AMCs. (B and C) LNCaP cells were plated in soft agar with the solvent DMSO or 50 μM of the indicated β-lactams. Cells were then cultured for 21 days without addition of new drug. The plates were scanned and a representative well from each treatment was selected for presentation (B). Colonies were quantified with an automated counter and presented as mean values from triplicate independent experiments. Error bars denote standard deviations (C).

and fragmentation (Fig. 7A). In addition, apoptosis-specific nuclear condensation was also observed in the remaining attached solid tumor (MCF-7, PCI-13, and DU-145) and transformed (VA-13), but not the normal (WI-38), cells (Fig. 7A). These results strongly suggest that lactam **12** induces apoptosis that lead to detachment preferentially in the tumor and transformed cells.

To confirm lactam **12**-mediated apoptotic cell death, in the same experiment, aliquots of both detached and attached cells of each line were combined and used for whole-cell extract preparation. This was followed by measurement of cell-free caspase-3/-7 activity. Consistent with the apoptotic nuclear changes (Fig. 7A), treatment of MCF-7, PCI-13, DU-145, and VA-13 cells with lactam **12** also increased levels of caspase-3/-7 activity by 11.0-, 10.2-, 5.2-, and 5.3-fold, respectively, *over* the control DMSO-treated cells (Fig. 7B). In addition, accompanying the lack of the detachment in normal WI-38 cells treated with lactam **12** (Fig. 7A), there was little or no induction of caspase-3/-7 activity observed in these cells (Fig. 7B). Taken together, these data further support the conclusion that lactam **12** is able to induce apoptotic cell death preferentially in tumor and transformed *over* the normal cells.

4. Discussion

Developing novel anticancer drugs that induce apoptosis in tumor cells has long been a goal of cancer drug discovery research. Many of the drugs in current use focus on targeting dysregulated cell cycle and apoptosis programs in cancer cells [25]. We previously have shown that *N*-thiolated β-lactams cause DNA damage in tumor cells that leads to induction of apoptosis through p38 activation, cytochrome *c* release, and caspase activation [17]. Here we show that lactam **1** selectively induces apoptosis in human leukemic Jurkat T cells, but not nontransformed, immortalized human NK cells (Fig. 2). Furthermore, lactam **1** is capable of inducing Jurkat cell apoptosis at concentrations as low as 10 μM after 24-hr treatment (Fig. 2A).

Often, addition/substitution of groups on a molecule leads to development of more potent drugs. In order to determine whether structural changes to lactam **1** could produce a more potent tumor cell death inducer, analogs of lactam **1** were synthesized (Fig. 1A). Substitutions of the –Cl group with other halogens of higher atomic mass (–Br, –I) did increase the efficacy of the compound. In contrast, substitution with a lower atomic mass halogen (–F) or a hydrogen (H) atom had a concomitant decrease in cell death induction (Fig. 1). Lactam **12**, containing an $-NO_2$ substituent, proved to be a highly active compound and induced 93–100% of cell death at 50 μM vs. 52% of cell death by lactam **1** at the same concentration (Fig. 1B). Furthermore, lactam **12** was superior to lactam **1** at inducting apoptosis in human Jurkat T cells because lactam **12** can induce the same amount of PARP cleavage at a lower concentration than lactam **1** (Figs. 3 and 4). Additionally, lactam **12** at 25 μM was able to exert its cell death-inducing effect at as early as 3 hr (Fig. 4A and B). We also found that lactam **12** had greater potency than lactam **1** when used in human prostate cancer cells to activating caspase-3/-7 and inhibiting colony formation (Fig. 6). Similar to our previous results with lactam **1** [17], we found that lactam **12** induces apoptosis in several solid tumor cell lines (e.g. MCF-7, PCI-13, DU-145) in a caspase-dependent manner (Fig. 7). Due to lack of caspase-3 in MCF-7 cells, it was believed that lactam **12**-mediated MCF-7 cell death was associated with caspase-7 activity (Fig. 7). Additionally, like lactam **1**, lactam **12** was also able to selectively induce apoptosis in human leukemic Jurkat T cells *over* nontransformed,

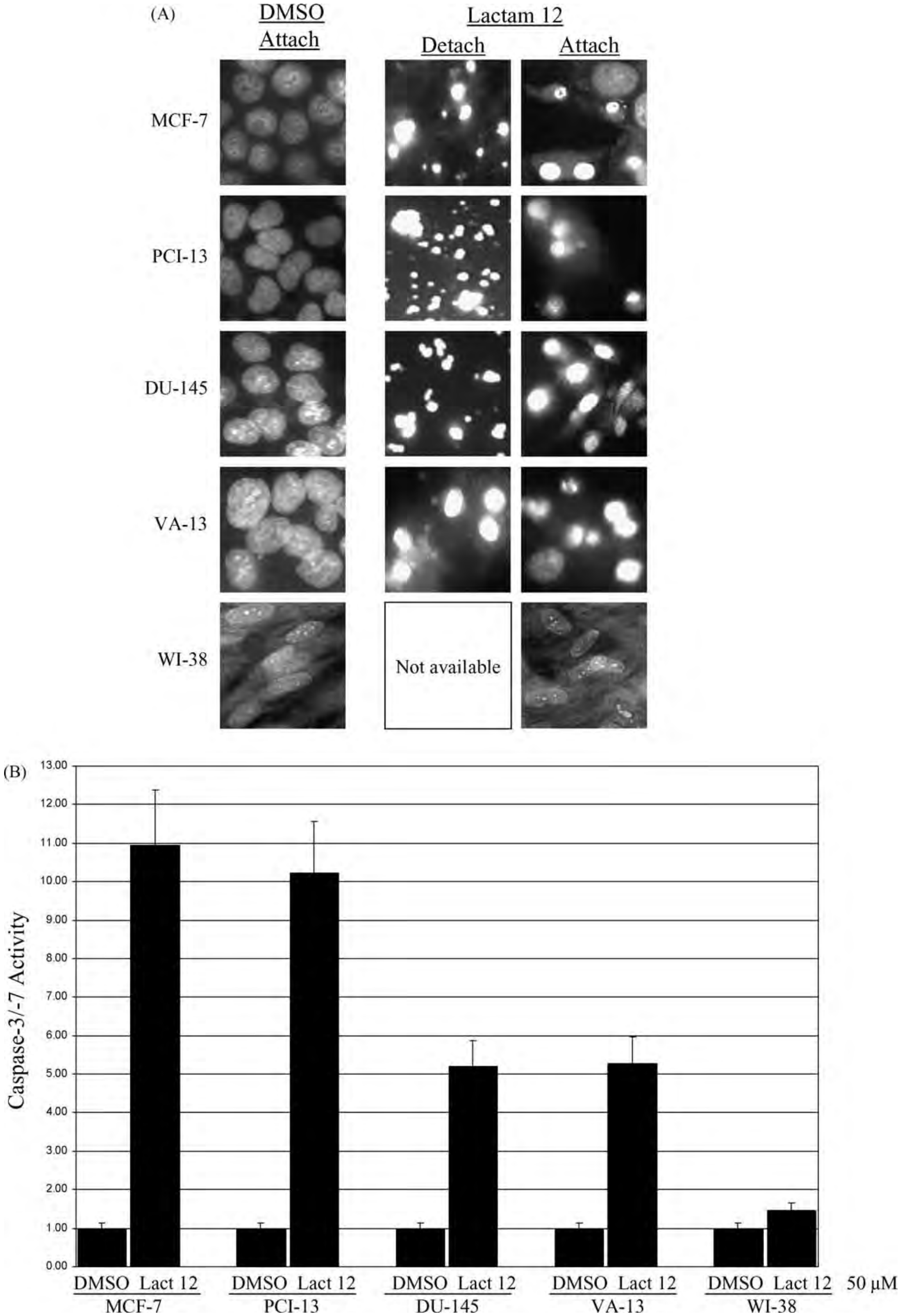

Fig. 7. Lactam **12** induces apoptosis and caspase activation in different solid tumor cell lines and SV40-transformed but not normal cells. (A) Nuclear staining assay. MCF-7, PCI-13, DU-145, VA-13, and WI-38 cells were treated with 50 μM lactam **12** or DMSO for 48 hr, followed by collection of both detached and attached cell populations. After lactam **12** treatment, ~60% of MCF-7 and PCI-13 cells and ~50% of DU-145 and VA-13 cells became detached, whereas <5% were detached from each of these cell lines after DMSO treatment. No detachment was found in WI-38 cells after each treatment. Both detached and attached cell populations were stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology. (B) Cell-free caspase-3/-7 activity assay. Aliquots of the above detached and attached cells of each line were combined for whole-cell extraction. Cell-free caspase-3/-7 activity was then determined by incubating each whole-cell extract with caspase-3/-7 substrate and measuring free AMCs. Error bars denote standard deviations. Similar results were obtained in three independent experiments.

immortalized human NK cells (Fig. 3). Also, lactam **12** was able to selectively induce apoptotic cell death in simian virus 40-transformed, but not the parental normal, human fibroblasts (Fig. 7). The molecular mechanism for the enhanced activity in lactam **12** remains unknown. One interpretation is that the presence of $-NO_2$ group in this drug increases its binding to the cellular target(s). Alternatively, this drug might have increased uptake rates by the cells.

The mechanism of action of many chemotherapeutic drugs is through DNA damage and then subsequent apoptosis induction in tumor cells [26]. As mentioned above, we have recently shown that lactam **1** is capable of inducing apoptosis after DNA damage [17]. In the present study, we show, by TUNEL assay, that lactam **12** also causes DNA damage in ~70% of cells just after 3-hr treatment (Fig. 5B). At this time, there was only 2% cell death (Fig. 5A), suggesting that the DNA damage occurs much earlier than apoptotic cell death. However, apoptotic cells increased at later time points with increased TUNEL-positive cells (Fig. 5A and B). This result is consistent with our previous study [17] and several other studies that have shown that several traditional chemotherapeutic drugs or DNA-damaging agents cause DNA strand breaks that trigger apoptotic cell death [27,28].

Malignant transformation of a cell can lead to tumor formation and metastasis. The desired effect of any anticancer drug is to inhibit tumor growth and formation *in situ*. Soft agar colony forming assay is an assay that has been developed to mimic tumor cellular growth in tissue. We hypothesized that the *N*-thiolated β-lactams that induce cell death should be able to inhibit colony formation in soft agar assay. Indeed, when LNCaP prostate cancer cells were cultured in the presence of lactam **1** or **12**, 91 and 100% inhibition of colony formation was observed, respectively, as compared to the solvent control (Fig. 6).

Based on our previous [17] and current studies, we propose that these *N*-thiolated β-lactams act by inducing DNA damage that leads to apoptosis preferentially in cancer and transformed *over* normal/nontransformed cells. Although it appears that the *N*-methylthio moiety is necessary for the cell death-inducing activity [17], addition of a larger group in the *ortho* position on the phenyl ring can also increase the effectiveness of the compound (Fig. 1). Our results strongly suggest the potential for developing this class of β-lactams into novel anticancer agents. Immediate future studies focusing on determining the molecular targets and chemical action of the *N*-thiolated β-lactams would help rational development of these compounds.

Acknowledgments

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STRUCTURE-ACTIVITY RELATIONSHIPS OF *N*-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS WITH C_3 SUBSTITUTIONS AND THEIR SELECTIVE INDUCTION OF APOPTOSIS IN HUMAN CANCER CELLS

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1. ABSTRACT

The development of novel anti-cancer drugs that induce apoptosis has long been a focus of drug discovery. Beta-lactam antibiotics have been used for over 60 years to fight bacterial infectious diseases with little or no side effects observed. Recently a new class of *N*-methylthiolated beta-lactams has been discovered that have potent activity against methicillin resistant *Staphylococcus aureas*. Most recently, we determined the potential effects of these *N*-thiolated beta-lactams on tumorigenic cell growth and found that they are apoptosis-inducers in human cancer cell lines. In the current study, we further determined the effects of the substitution of the *O*-methyl moiety on C_3 and stereochemistry of the beta-lactams on the anti-proliferative and apoptosis-inducing abilities. We have found that lactam 18, in which C_3 is substituted with an acrylate ester group, is a very effective proliferation inhibitor against human premalignant and malignant breast, leukemic, and simian virus 40-transformed fibroblast cells. Generally speaking, increasing the size of the moiety on C_3 decreases its anti-proliferation potency, possibly indicating steric hindrance with the cellular target or decreased permeability through the cell membrane. We also found that the stereochemistry of the beta-lactams plays an important role in their potency. The 3S,4R isomers are more effective than their enantiomers (3R,4S), suggesting that 3S,4R configuration is more favorable for target interaction.

2. INTRODUCTION

Selectively targeting tumorigenic cells *versus* normal cells is a primary goal in anti-cancer drug discovery. Small molecules with apoptosis-inducing ability have great potential to be developed into novel chemotherapeutic drugs because of the ease of synthesis and structural manipulation (1-3). Initiation, commitment, and execution are the three fundamental steps of apoptosis (4). Several apoptotic stimuli, such as irreparable DNA damage, signal to activate the initiator caspases (*e.g.* caspases-8/10), which in turn activate downstream effector caspases (*e.g.* caspases-3/7). The effector caspases can also be activated through the release of mitochondrial proteins, such as cytochrome *c* (5). It is generally believed that proteolytic cleavage of a variety of intracellular substrates by effector caspases leads to apoptosis (6-9).

One particularly important class of small molecule drugs, the beta-lactam antibiotics, have played an essential role in treating bacterial infections without causing toxic side effects in the host for the past 60 years. Sir Alexander Fleming first coined the name "penicillin" in 1928 after his discovery that molds from the *Penicillium* genus secrete powerful antimicrobial compounds, called beta-lactams (10). X-ray crystallography revealed that penicillin is a thiazolidine ring fused to a four membered beta-lactam ring (11). The beta-lactams are powerful and potent inhibitors of bacterial growth and many different moieties of bicyclic beta-lactams have been isolated or synthesized since the discovery of penicillin (12). There are several classes of bicyclic beta-lactams that possess antibacterial properties, including the penams, penems, carbapenems, cephalosporins, and clavulanic acids (10).

A novel class of beta-lactams ~~were~~ was discovered by the Squibbs and Takeda laboratories in 1981, which have an *N*-sulfonic acid group attached directly to the nitrogen in the lactam ring (13 , 14). The term "monobactam" was coined for these lactams, which have a flexible monocyclic ring~~,~~ and lack the carboxylic acid moiety, yet still retain a high bactericidal potency. Recently, a structurally related family of *N*-thiolated compounds, termed *N*-methylthio beta-lactams, ~~were~~ was found to inhibit growth of *Staphylococcal* and methicillin-resistant *S. aureus* (MRSA) (15-17). Additionally, we have shown that these *N*-methylthio beta-lactams possess potent anti-proliferative properties, and are capable of inducing DNA strand breakage, inhibiting DNA replication, and inducing apoptosis in a time- and concentration-dependent manner when tested in several human cancer, but not normal cell lines (18 , 19).

In this study, we screened several additional *N*-thiolated beta-lactams with substitutions made to the *O*-methyl moiety of carbon 3 (C_3) for their structure-activity relationships and found that increasing the size of the C_3 substitution results in decreased anti-proliferative activity in human breast cancer cells. Additionally, increasing the size of the C_3 substituent may interfere with cellular uptake. We identified one particularly active lactam (lactam **18**), which possesses an acrylate ester moiety off of C_3, for further study. Lactam **18** induces caspase-3 activation and apoptosis, associated with increased Hsp70 protein expression and p38 phosphorylation. We have also found that the stereochemistry plays an important role in the activities of *N*-thiolated beta-lactam antibiotics, including anti-proliferation, S/G_2/M cell cycle arrest, and apoptosis induction. The 3S,4R-configured [(+)] isomers of lactam **18** and ~~another~~ lactam **19** are ~~both~~ more potent than their 3R,4S-configured isomers or the racemic mixtures. Furthermore, these (+)-lactams are more efficacious than racemic lactam **1**, which was identified from our previous studies (18). These effects of beta-lactams were found mainly in cultured human cancer and transformed cells, but not in normal/non-transformed cells. These data indicate that further study of *N*-thiolated beta-lactams in the treatment of cancers is warranted.

MATERIALS AND METHODS

3.1. Reagents

Fetal Bovine Serum was purchased from Tissue Culture Biologicals (Tulare, CA). Mixture of penicillin-streptomycin-L-glutamine, RPMI, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 (1:1) medium, horse serum, MEM non-essential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), epidermal growth factor (EGF), sodium bicarbonate, hydrocortisone, cholera enterotoxin, bovine insulin, propidium iodide and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies to actin, monoclonal antibodies to HSP70 and p-p38, and anti-goat and anti-mouse IgG-horseradish peroxidase were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). CaspACE FITC-VAD-FMK marker was purchased from Promega (Madison, WI). Fluorogenic peptide substrate Ac-DEVD-AMC (for caspase-3/-7 activities) was obtained from Calbiochem (San Diego, CA).

3.2. Synthesis of beta-lactams

The beta-lactam analogs (Figure 1) were prepared as racemates and enantiomers (with *cis* stereochemistry) using a procedure described previously (15 , 16).

3.3. Cell culture, protein extraction, and Western blot assay

Human leukemic Jurkat T cells and natural killer YT cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Further supplementation with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM non-essential amino acids solution was added to YT cells. Human breast cancer MCF-7 cells, normal (WI-38) and SV-40 transformed (VA-13) human fibroblasts cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. Premalignant MCF10AT1Kcl.2 transformed human breast cells (20) were cultured in DMEM/F12 (1:1) supplemented with 10 μg/ml bovine insulin, 100ng/ml cholera enterotoxin, 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 29 mM sodium bicarbonate, 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2. A whole-cell extract was prepared and Western blotting was performed as described previously [18].

3.4. Cellular proliferation assay

The MTT assay was used to determine the effects of beta-lactams on overall proliferation of tumor cells. Cells were plated in a 96-well plate and grown to 70-80% confluency, followed by addition of each compound at an indicated concentration for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37ºC for 4 hours to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 μl of DMSO was added, followed by colorimetric analysis using a multilabel plate reader at 560 nm (Victor[3]; Perkin Elmer). Absorbance values plotted are the mean from triplicate experiments.

3.5. Trypan blue assay

The trypan blue dye exclusion assay was performed by mixing 20 μl of cell suspension with 20 μl of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

3.6. Cell cycle assay

Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted, and washed twice with PBS. Cells (5×10^6) were then suspended in 0.5 ml of PBS, pipetted, and fixed in 5 ml of 70% ethanol for at least 2 h at -20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50 µg propidium iodide, 1 mg RNase A, and 1 mg of glucose per ml of PBS) and incubated at room temperature for 30 min before flow cytometry analysis. The cell cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M DNA judged by propidium iodide staining.

3.7. Caspase-3 activity assay

To measure cell-free caspase-3 activity, whole cell extracts (30 μg) from untreated or treated cells were incubated with 20 μM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 30 min at 37°C in 100 μl of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a Victor[3] Multilabel plate reader™ (Perkin Elmer) as described previously [18].

3.8. Immunostaining of apoptotic cells

Immunostaining of apoptotic cells was performed by addition of the FITC-VAD-FMK marker and visualized on an Axiovert 25 microscope (Zeiss; Thornwood, NY). Briefly, cells were grown to ~80% confluency in 60 mm dishes, and then treated under conditions described in the figure legends. Detection of caspase activity was determined according to the manufacturer's protocol with a few modifications. Briefly, total cell population was collected and incubated with a 10 μM FITC-VAD-FMK for 20 min in the dark. Cells were then centrifuged at 300 xg for 3 minutes, washed 3X in PBS, and then resuspended in 50 μl PBS. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. Apoptotic cells were quantified by counting the number of apoptotic cells *over* the total number of cells in the same field.

3.9. Nuclear staining

After each drug treatment, both detached and attached populations of VA-13 and WI-38 lines were stained with Hoechst 33342 to assess apoptosis. Briefly, cells were washed 2X in PBS, fixed for 1 h with 70% ethanol at 4ºC, washed 3X in PBS, and stained with 50 μM Hoechst for 30 min in the dark at room temperature. Detached cells were plated on a slide and attached cells were visualized on the culture plate with a fluorescent microscope at 10X or 40X resolution (Zeiss, Thornwood, NY). Images were obtained using an AxioVision 4.1 and adjusted using Adobe Photoshop 6.0.

4. RESULTS

4.1. Structure-activity relationship analysis of *N*-thiolated beta-lactams

We have previously shown that lactam **1**, which contains an *O*-methyl moiety at carbon-3 (C_3) of the beta-lactam ring, induces apoptosis in a relatively selective manner in tumor and transformed cells, but not normal or non-transformed cell lines (18). More than 35 *N*-thiolated beta-lactam analogs were then screened using an MTT assay in breast cancer MCF-7 cells to assess their anti-proliferative potency compared to lactam 1. Several of these analogs with substitutions to the *O*-methyl group at C_3 (Figure 1) were then selected for further structure-activity relationship (SAR) studies (Figure 2). MCF-7 cells were treated with 50 μM of selected lactams for 24 h, followed by MTT assay. Lactam 18, which possesses an ester moiety at C_3, is twice as potent as lactam 1 (Figure 2). Additionally, it was found that as the C_3 group increased in size, the effectiveness to inhibit proliferation decreased. For instance, increasing the size of the halogen, from –Cl to –I (lactam 13 *versus* lactam 14), lead to a 2-fold decrease in potency (Figure 2). A similar observation was made in comparing the bioactivities of C_3-sulfonated lactams 16 and 17. Mesyl lactam 16 inhibited 57% of MCF-7 cell growth, while dansyl lactam 17 has much less effect on inhibiting proliferation (Figure 2). A possible explanation may be that lactam 17 with the large dansyl group is incapable of crossing the cell membrane. Although lactam 15 with an N_3 group at C_3 is less potent than lactam 14 with I at C_3, as predicted, it is yet unclear why lactam 15 is less potent than mesyl lactam 16 (Figure 2). The order of potency is determined as follows: Lactam 18 > 1 > 16 ≥ 13 > 14 > 17 > 15.

4.2. Lactam 18 is more potent than lactam 1 at inducing apoptosis associated with Hsp70 expression and p38 phosphorylation

We decided to focus on lactam **18** due to its increased anti-proliferative potency *over* lactam **1** (Figure 2). To determine whether lactam 18 is capable of inducing apoptosis, we treated leukemia Jurkat T cells with 20 μM lactam 18 for 24 h, using lactam 1 as a control. A fluoroscent marker specific for activated caspases was then added to the cells and then visualized by fluorescence microscopy (Figure 3A). Lactam 18 displayed greater apoptosis-inducing activity than lactam 1 (Figure 3A).

Microarray analysis showed up-regulation of many genes by lactam 1 treatment, including *HSP70* (manuscript in preparation). To confirm this finding, Jurkat T cells were treated with lactam 1 and lactam 18 for 16 h at either 25 or 50 μM. It was found that Jurkat T cells had increased Hsp70 protein expression after treatment of both lactams; lactam 18 induced a greater amount of Hsp70 expression at 25 μM than lactam 1 at the same concentration (Figure 3B). This is consistent with the idea that the beta-lactams induce a stress response in the cell, most likely due to their disruption of DNA synthesis (18).

We have previously reported that p38 MAP kinase activation is associated with β-lactam-induced apoptosis (18). Activation of p38 MAP kinase can trigger apoptosis following multiple stimuli, such as DNA damage (21 , 22). Western blot for phosphorylated (activated) pp38 reveals that treatment with 25 μM lactam 18 leads to an increase in pp38 levels (9.0-fold), compared to that of 25 μM lactam 1 (2.6-fold) (Figure 3B). Actin was used as a loading control.

4.3. (+)-Lactam 19 inhibits cellular proliferation more effectively than (-)-lactam 19

To determine if the stereochemistry has any bearing on the potency of beta-lactams, lactams 18 and 19 were synthesized in enantiomerically pure forms (Figure 1). Premalignant MCF-10AT1Kcl.2 breast cancer cells were treated with (+)-lactam 19 (3S,4R-configuration), (-)-lactam 19 (3R,4S-configuration), or racemic lactam 1 (as a control) for 24 h at indicated doses (Figure 4A). All of these lactams inhibited proliferation in a dose-dependent manner, with (+)-lactam 19 at 50 μM inhibiting 85% cell growth, (-)-lactam 19 inhibiting 56% and lactam 1 inhibiting 49% at the same concentration (Figure 4A). These results are similar to that from another experiment using malignant MCF-7 breast cancer cells (data not shown). Trypan blue incorporation also shows increased tumor cell killing with (+)-lactam 19 to that of (-)-lactam 19, 42% versus 26%, respectively, at 25 μM (Figure 4B).

4.4. beta-Lactams 18 and 19 induce tumor cell-selective apoptosis

We have previously shown that lactam 1 preferentially induces apoptosis in human cancer cells over normal, non-transformed cells lines (19). To determine if lactam 18 possessed a similar tumor cell-specific activity, human leukemic Jurkat T cells and immortalized, non-transformed natural killer cells (YT) were treated with lactam 1 and lactam 18, and the effects were determined. After 16 h treatment, it was found that lactam 18-treated Jurkat cells had a 19-fold increase in caspase-3 activity at 25 μM, compared to 9-fold induced by lactam 1 at the same concentration (Figure 5A). Both lactam 18 and lactam 1 had little or no apoptosis-inducing effects on the immortalized, non-transformed YT cells (Figure 5A).

To assess whether the isomers of lactam 19 (Figure 1) also have explicit activity against cancer cells, we treated Jurkat T cells and YT cells with (+)-lactam 19, (-)-lactam 19 and racemic lactam 1 (as a control), and determined the effects on apoptotic cell death. We found that only the Jurkat, but not YT, cells exhibited high levels of caspase-3 activity when treated with these beta-lactams (Figure 5B). Additionally, (+)-lactam 19 at 25 μM induced a 7.4-fold increase in caspase-3 activity compared to 5.5-fold increase by (-)-lactam 19 and 3.1-fold increase by lactam 1 at 25 μM (Figure 5B). Therefore, the 3S,4R-configured isomer, (+)-lactam 19, is more potent than (-)-Lactam 19 and racemic lactam 1.

To further confirm the effect of stereoselectivity on the apoptosis-inducing effects of beta-lactams, we synthesized an isomeric pair of lactam 18, 3S,4R-isomer [(+)-lactam 18] and 3R,4S-isomer [(-)-lactam 18] (Figure 1). Jurkat and YT cells were then treated with 50 μM (+)-lactam 18, (-)-lactam 18, racemic lactam 18 or lactam 1 for 24 h, followed by trypan blue dye exclusion assay (Figure 6A). (+)-lactam 18 induced much higher amount of cell death than its isomer (-)-lactam 18, 98% *vs.* 58%, respectively (Figure 6A). Interestingly, the racemic lactam 18 was almost equally potent to that of (+)-lactam 18 (Figure 6A). All lactam 18 compounds initiated more cell death than lactam 1 (Figure 6A). Again, it was found that normal, non-transformed YT cells did not undergo cell death after treatment with any of these beta-lactams (Figure 6A).

Another experiment using SV-40 transformed (VA-13) and normal (WI-38) human fibroblasts demonstrates again that (+)-lactam 18 is the more active isomer. A nuclear stain of VA-13 and WI-38 cell lines treated with 50 μM of each beta-lactam for 24 h reveals that there is a high degree of detachment and DNA condensation, characteristics indicative of apoptosis, in cells treated with (+)-lactam 18, racemic lactam 18 and lactam 1 (Figure 6B). (-)-lactam 18, on the other hand, showed decreased activity when compared with (+)-lactam 18 and racemic lactam 18. There was a very minor amount of cellular detachment observed in the normal WI-38 fibroblasts treated with (+)-lactam 18 (data not shown), supporting that these beta-lactams selectively kill transformed VA-13 cells (Figure 6B).

4.5. (+)-Lactam 18 induces S/G2/M cell cycle arrest

We have previously shown that beta-lactams decrease G_1 population, associated with DNA damage (18). To further investigate the cause of apoptosis after *N*-methlythio beta-lactam treatment, analysis of cell cycle changes were performed on an exponentially growing cell population (Table 1). As a control, lactam 1 was found to decrease G_1 phase DNA content by 6% after 6 h incubation (Table 1). Racemic lactam 18 had a very similar effect on cell cycle as racemic lactam 1 (Table 1). However, when cells were treated with (+)-lactam 18, there was a 15% decrease in G_1, demonstration that the 3S,4R configuration has increased growth-inhibitory activity (Table 1).

5. DISCUSSION

Currently, many anticancer therapies, from radiation treatment to chemotherapeutic agents, are very toxic. Therefore, drug discovery for anticancer therapy is as concerned with selectivity of normal *versus* cancer tissues, as it is the potency of the therapy itself. Antibiotic therapies have typically used the unique molecular targets of microbes in order to avoid toxicity to the patient during treatment. Recently we have shown that some of these compounds possess anti-proliferation activity in human tumor cells (18). Thus, these compounds that are already known to be essentially non-toxic to humans may be anticancer agents as well. Of particular interest are the *N*-thiolated beta-lactams, which we have previously found to be potent against MRSA (17). Additionally, we found these compounds act as potential anticancer agents through S-phase arrest, DNA damage, and apoptosis induction (18). These compounds are also able to selectively induce apoptosis in cancerous *over* normal cells (19).

This novel class of *N*-thiolated beta-lactams possesses potent anti-cancer activity, which is directly related to the nature of the substituents on each of the four ring sites. We have previously reported on the effects of additions/substitutions to the *N*-thio group and aryl ring (18 , 19). The work reported here is a further characterization of the SAR between the various substitution groups on beta-lactam ring. At the core of the beta-lactam molecule is a four-membered ring that is substituted at each position (Figure 1). Each of the positions, we have previously published, plays a role in the potency of the compound. Position 1 is the N-methythio position and changes at this position that either eliminate the methylthio moiety or lengthen the chain result in decreased potency (18). Position 2 is substituted with a benzene ring and changes here also effect potency (19). Physical position on the ring with regards to ortho-, para-, or meta substitution as well as the nature of the substituent affected the potency dramatically [for details see (19)]. Position 4, which is substituted with a double-bonded oxygen, is the "backbone" of the beta-lactam and therefore cannot be changed without losing the general beta-lactam framework (10 , 23). The work presented here examines the SAR at position 3 and completes the survey of each position of the four-membered ring.

We determined that the size and polarity of the group at C_3 is important for the~~ir~~ lactam's activity. As these C_3 substituents increase in size or in polarity, the efficacy of the compound seems to drop. For example, replacing the chloro (Cl) moiety of lactam 13 for azido (N_3), lactam 15, decreases the anti-proliferative activity from 75 to 5% respectively (Figure 2). However, a simple single atom change in the same period (Cl to I; lactam 13 *vs.* lactam 14) seems to result in only a partial increase in potency. Similarly, the potencies of C_3-sulfonated compounds 16 and 17 can be directly attributed to their C_3 substitutions: lactam 17 with its large, polar dansyl moiety has significantly diminished activity compared to its smaller, less polar mesyl analog, lactam 16 (Figure 2). Likewise, lactam 16 seems to be similar in or slightly less potent than lactam 14, indicating that the size of the substituent may be slightly more significant than the overall charge (Figure 2). Lactam 18 displays very potent activity with its acrylate ester off C_3. This may indicate that these substitutions may affect the capability of these compounds to cross the cell membrane.

Of primary importance in anticancer drug research is that the compound being investigated demonstrate selectivity between normal cells and tumor cells. Cytotoxic agents are less desirable than those compounds that can differentially activate apoptosis in cancer cells *vs.* tumor cells. Previously we have reported that lactam 1-induced apoptosis is caspase-dependent and associated with cytochrome c release (18). Here we show that lactam 18-induced apoptosis is also caspase-dependent (Figure 5A). However, the efficacy to induce apoptosis by lactam 18 is much improved over lactam 1 (Figure 3) and that the apoptosis induced by lactam 18 is tumor cell-specific (Figure 5A). beta-Lactams cause DNA strand breakage and subsequent cell cycle arrest (18). Our microarray studies show a 3.5-fold increase in *HSP70* expression in Jurkat T cells treated with lactam 1 (data not shown). Increased expression of Hsp70 protein (Figure 3B) indicates that treatment with lactam 18 induces a stress response in leukemic Jurkat T cells. Another important molecular event in lactam-induced apoptosis is the increase in p38 phosphorylation. Abrogation of pp38 activity with a specific inhibitor (PD169316) leads to tumor cell survival (18). Not only is lactam 18 capable of inducing p-38 activation, it is capable of inducing a greater amount of pp38 levels at 25 μM compared to 50 μM of lactam 1 (Figure 3B).

Stereochemistry can play an important role in the efficacy of a particular compound. Often only one of the isomers displays a significant selectivity for the molecular target while the other can cause adverse side effects (24-26). Here we find that two 3S,4R-configured beta-lactam compounds, (+)-lactam 18 and (+)-lactam 19, do have a higher potency than their 3R,4S enantiomers or a racemic mixture. Specifically, (+)-lactam 19 has greater anti-proliferation and cell death-inducing activities than both (-)-lactam 19 and the racemic lactam 1 (Figure 4). (+)-Lactam 19 triggers an equivalent amount of capase-3 activation at half the concentration of lactam 1 and this activity again is tumor cell-selective (Figure 5). Additionally, another isomer, (+)-lactam 18, displays a similar potency to (+)-lactam 19 while still retaining the tumor cell-selectivity (Figures 5B and 6).

A vast amount of anti-cancer research is ongoing to develop apoptosis-inducing drugs. While the molecular targets and chemical actions of *N*-thiolated beta-lactams are not fully characterized, we believe that the compounds possess great potential for chemotherapeutic drug development. These antibiotics compounds are predicted to have little to no effect on normal cells, supported by our results. Thus, the anti-tumor potential and expected lack of toxicity of these beta-lactams makes them excellent candidates for anticancer drug development. Our ongoing studies focus on identifying the molecular interactions of beta-lactams in human cancer cells and their anti-tumor activities *in vivo*.

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Figure 1. Chemical structures of *N*-methylthiolated beta-lactams.

Figure 2. Structure-activity relationship (SAR) analysis of *N*-thiolated beta-lactams. MCF-7 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50 μM beta-lactam for 24 h. Cells were then incubated with 1 mg/ml MTT for 3 h and proliferation rates were determined using a muli-label plate reader (Victor3, Perkin Elmer; ±SD).

Figure 3. Lactam **18** induces caspase activity associated with Hsp70 expression and p38 phosphorylation. **A**. Jurkat T cells were treated with 20 μM lactam **1** or lactam **18** for 24 h. Following the treatment, the cells were then incubated with a FITC-conjugated marker that binds to activated caspases. Cell suspension was then transferred to glass slides in the presence of Vector Shield mounting medium with DAPI. Images were captured using AxioVision 4.1 and adjusted using Adobe Photoshop 6.0 software. **B**, Jurkat cells treated with 25 or 50 μM of lactam **1** or lactam **18** for 16 h, followed by Western blot analysis using specific antibodies to HSP70, p-p38, and Actin. Data shown are representative from three independent experiments.

Figure 4. (+)-Lactam **19** effects proliferation and cell death in a dose-dependent manner. **A**, MCF10AT1Kcl.2 cells were plated in a 96-well plate and grown to 70-80% confluency followed by addition of 50 μM of indicated beta-lactams for 24 h. Cells were then incubated with 1 mg/ml MTT for 4 h and proliferation rates were determined using a muli-label plate reader (Victor3, Perkin Elmer; ±SD). **B**, Jurkats T cells treated with lactam **1**, (+)-lactam **19,** (-)-lactam **19** at indicated doses and assayed for cell death by trypan blue incorporation (±SD).

Figure 5. beta-Lactams induce apoptosis in a tumor cell-specific manner. **A**, Jurkat T and YT cells were treated with lactam **1** and lactam **18** at indicated concentration for 16 h, followed by measurement of cell-free caspase-3 activity by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs. **B**, (+)-lactam **19** is 2-fold more potent that lactam **1** at inducing apoptosis in a tumor cell specific manner. Jurkat T and YT cells were treated for 24 h with 25 and 50 μM of lactam **1** *versus* 25

μM of (+)-lactam **19** and (-)-lactam **19**. Cell-free caspase-3 activity was then determined by incubating whole cell extracts with caspase-3 substrate and measuring free AMCs.

Figure 6. (+)-Lactam **18** induce apoptosis selectively in tumorigenic cells. **A**, Leukemic Jurkat T and non-transformed YT cells were treated with lactam **1** or isomers of lactam **18** at 50 μM for 24 h. Cell death is given as a percent of dead cells over total cell population (±SD). **B**, Both detached and attached VA-13 and WI-38 fibroblast cell populations were collected and stained with the nuclear staining dye Hoechst 33342. Each sample was then analyzed by fluorescence microscopy for nuclear morphology.

Table 1. Cell cycle analysis of asynchronous Jurkat T cells treated with beta-lactams at 50 μM for 6 h.[1]

	No treat	Lactam **1**	Lactam **18**	(+)-Lactam **18**
% G_0/G^1	42	36	36	27
% S	42	38	41	46
% G2/M	16	26	23	27
% G0/G1 Δ[2]	--	-6	-6	-15

[1]The cell cycle distribution was measured as the percentage of cells that contain G_1, S, G_2 and M DNA ($G_1/S/G_2/M$ = 100%).
[2]The percent change from control cells (no treat) is shown as % Δ.

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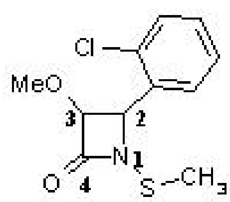
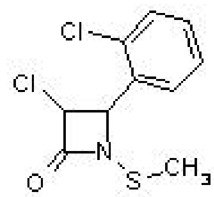


Lactam 1
racemic



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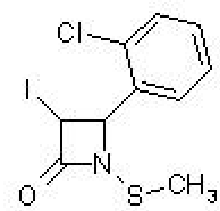
racemic



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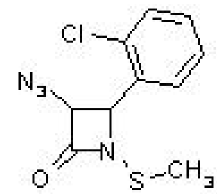

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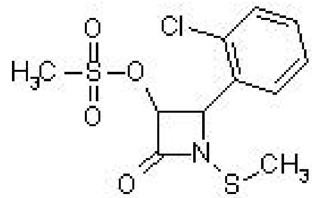

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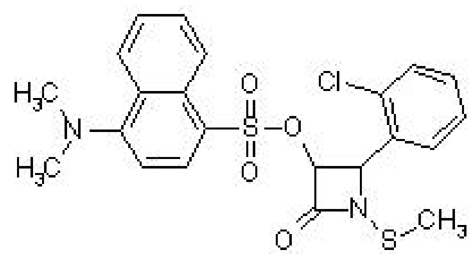
racemic



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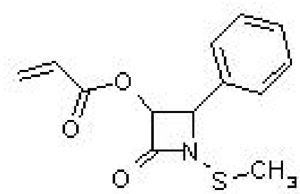
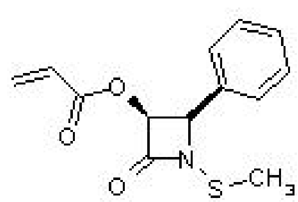
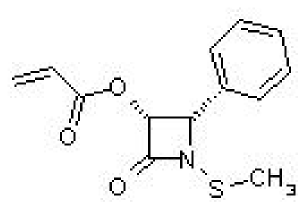
racemic



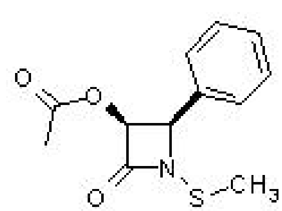
Lactam 18
racemic



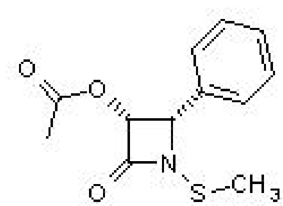
Lactam (+)-18
3S, 4R-configuration



Lactam (-)-18
3R, 4S-configuration



Lactam (+)-19
3S, 4R-configuration



Lactam (-)-19
3R, 4S-configuration

Figure 1

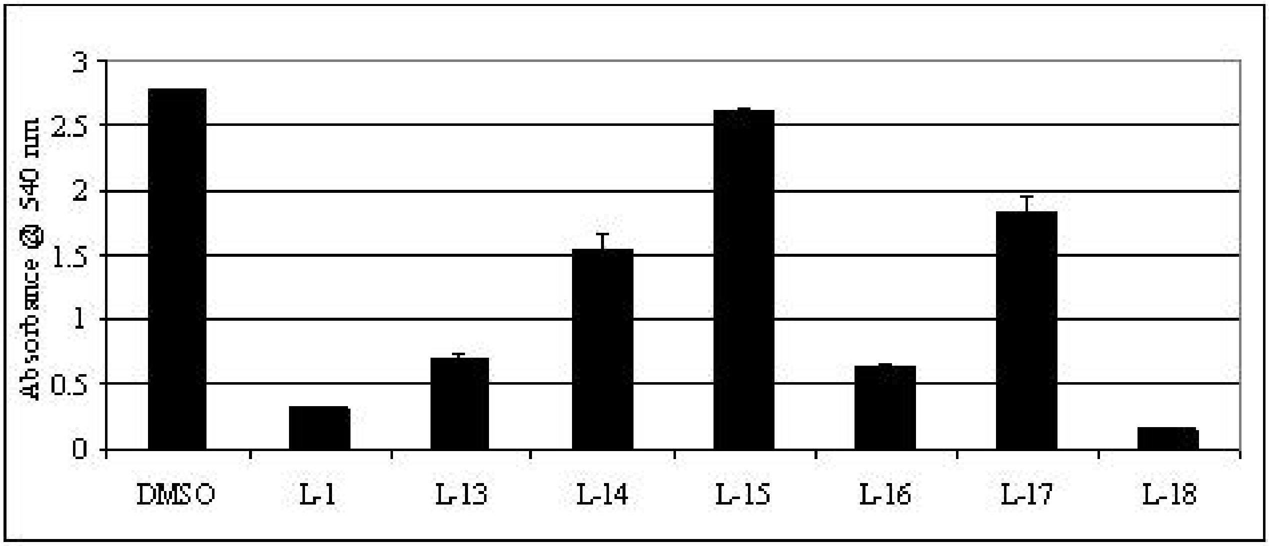


Figure 2

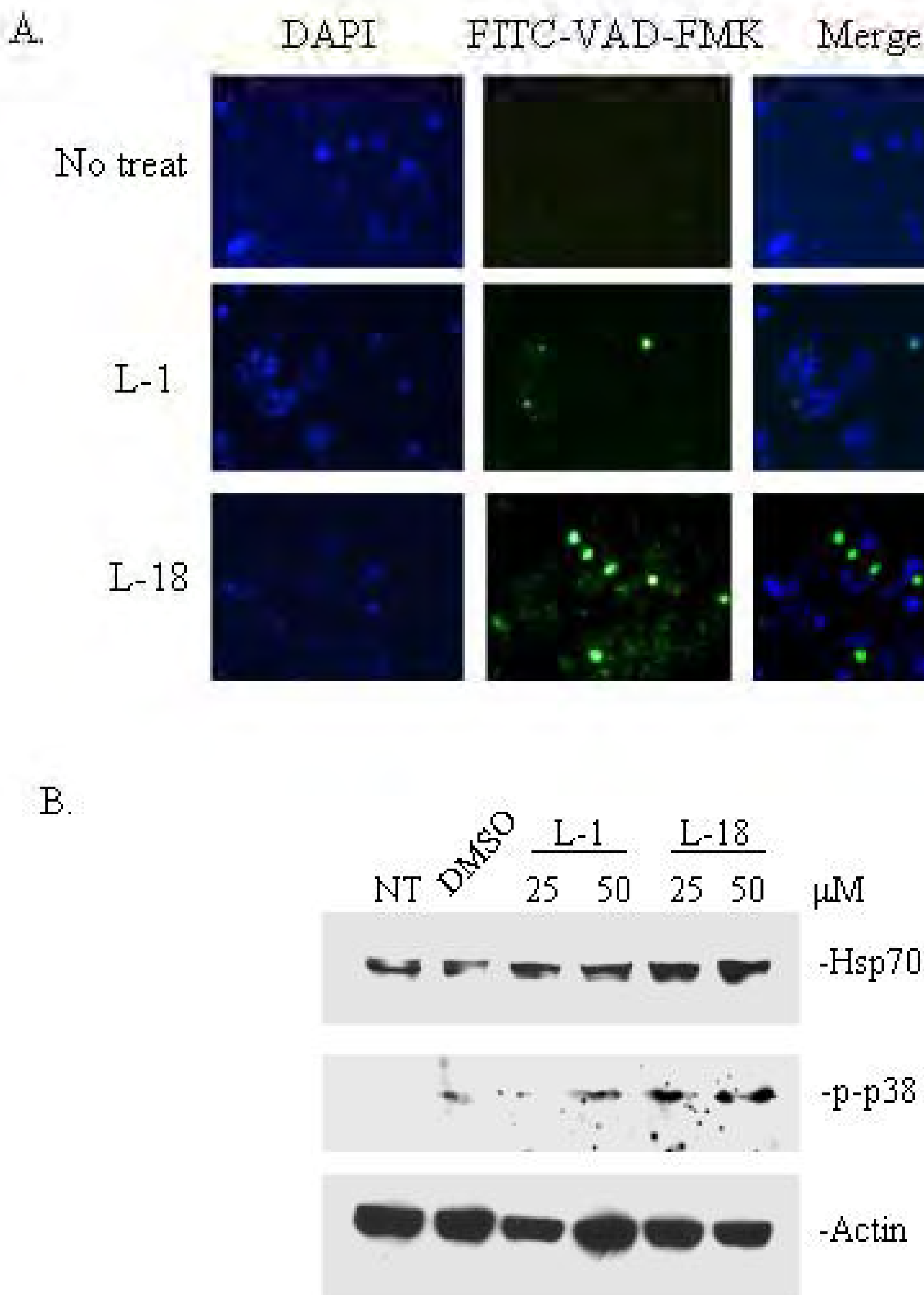
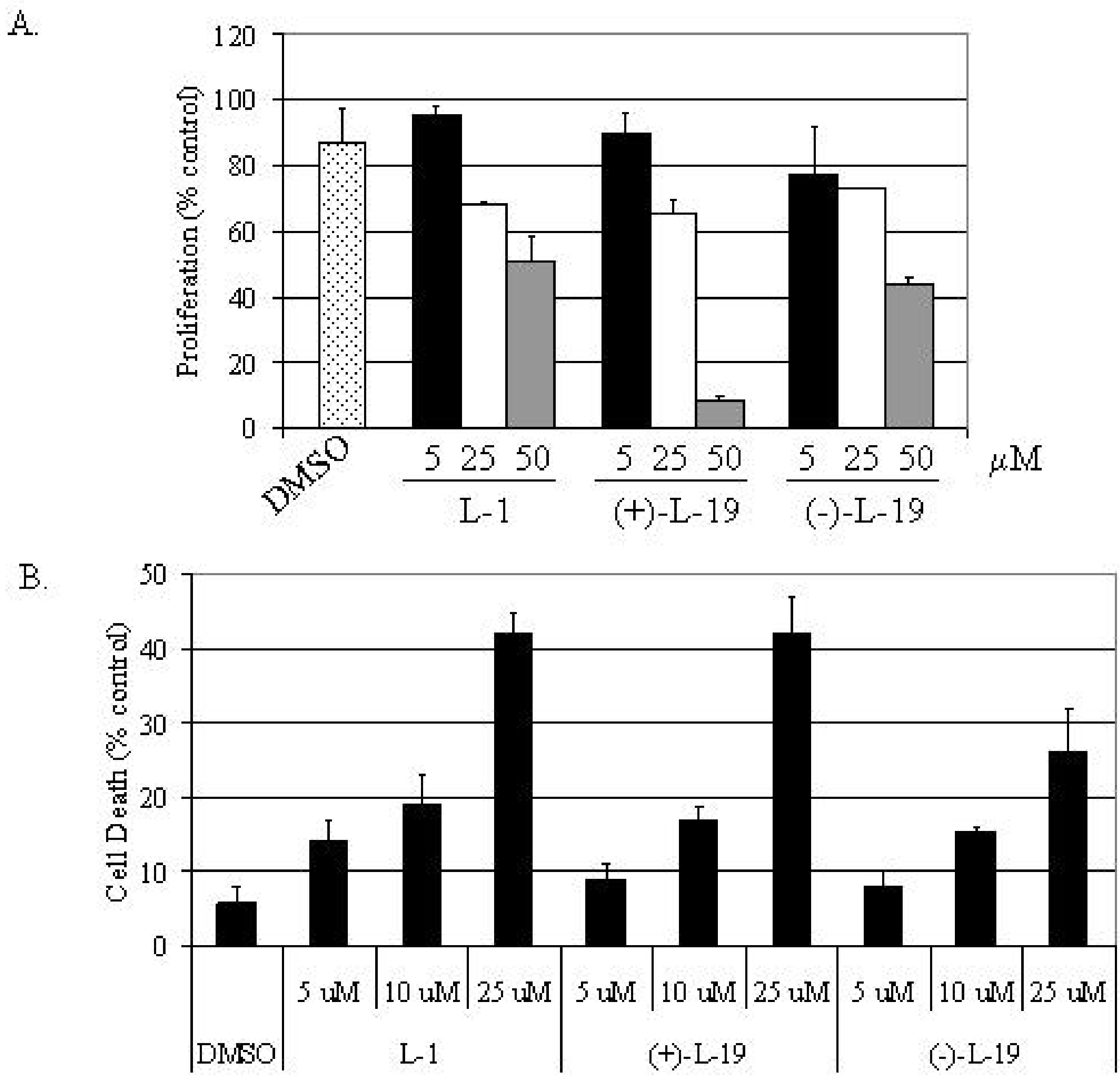

A.
DAPI
FITC-VAD-FMK
Merge
No treat
L-1
L-18
B.
NT
DMSO
L-1
L-18
25
50
25
50
μM
-Hsp70
-p-p38
-Actin

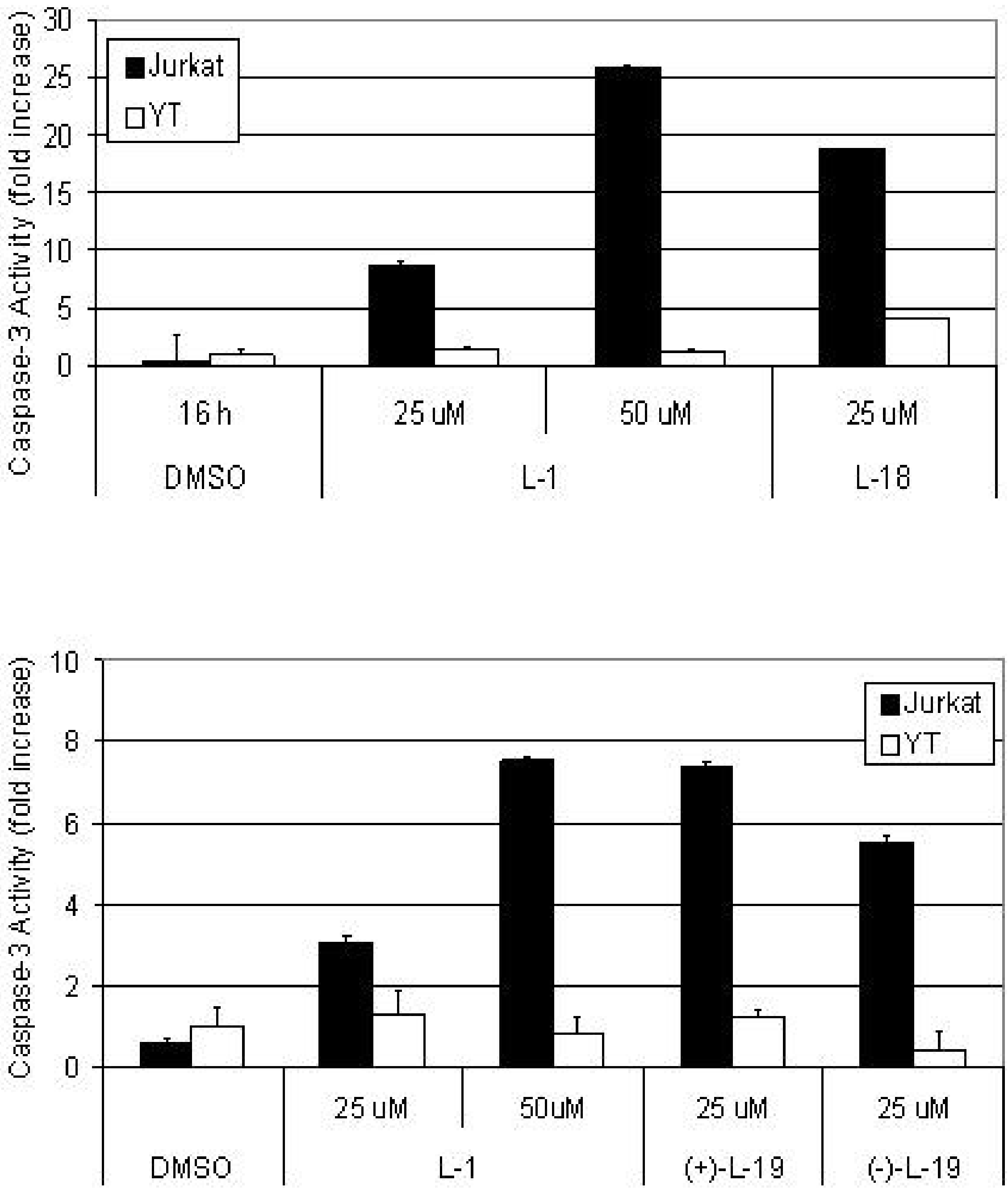
Figure 3



A.
120
100
80
60
40
20
0
Proliferation (% control)
DMSO
5 25 50
5 25 50
5 25 50
μM
L-1
(+)-L-19
(-)-L-19
B.
50
40
30
20
10
0
Cell Death (% control)
5 uM
10 uM
25 uM
5 uM
10 uM
25 uM
5 uM
10 uM
25 uM
DMSO
L-1
(+)-L-19
(-)-L-19

Figure 4

A.



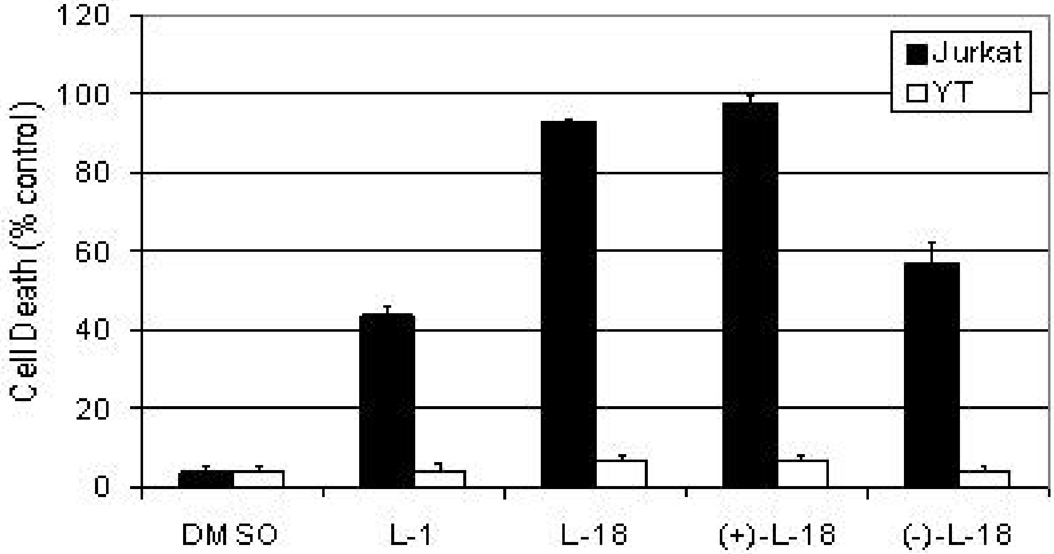
Caspase-3 Activity (fold increase)
30
25
20
15
10
5
0
Jurkat
YT
16 h
25 uM
50 uM
25 uM
DMSO
L-1
L-18

B.

Caspase-3 Activity (fold increase)
10
8
6
4
2
0
Jurkat
YT
25 uM
50uM
25 uM
25 uM
DMSO
L-1
(+)-L-19
(-)-L-19

Figure 5

A.


120
100
80
60
40
20
0
Cell Death (% control)
Jurkat
YT
DMSO
L-1
L-18
(+)-L-18
(-)-L-18

B.

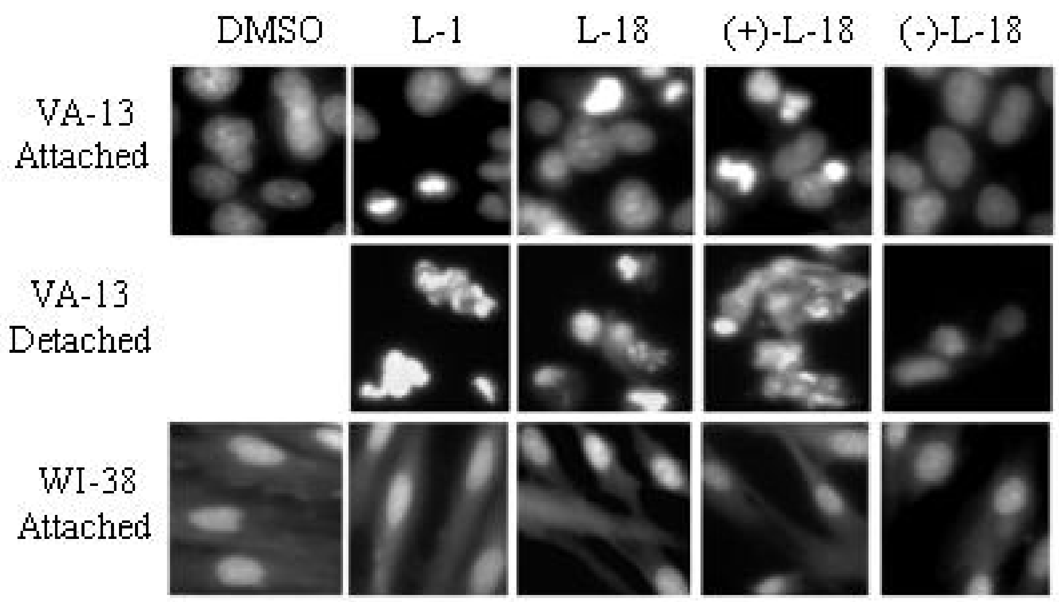

DMSO
L-1
L-18
(+)-L-18
(-)-L-18
VA-13 Attached
VA-13 Detached
WI-38 Attached

Figure 6

Research article

Open Access

Clioquinol and pyrrolidine dithiocarbamate complex with copper to form proteasome inhibitors and apoptosis inducers in human breast cancer cells

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Abstract

Introduction A physiological feature of many tumor tissues and cells is the tendency to accumulate high concentrations of copper. While the precise role of copper in tumors is cryptic, copper, but not other trace metals, is required for angiogenesis. We have recently reported that organic copper-containing compounds, including 8-hydroxyquinoline-copper(II) and 5,7-dichloro-8-hydroxyquinoline-copper(II), comprise a novel class of proteasome inhibitors and tumor cell apoptosis inducers. In the current study, we investigate whether clioquinol (CQ), an analog of 8-hydroxyquinoline and an Alzheimer's disease drug, and pyrrolidine dithiocarbamate (PDTC), a known copper-binding compound and antioxidant, can interact with copper to form cancer-specific proteasome inhibitors and apoptosis inducers in human breast cancer cells. Tetrathiomolybdate (TM), a strong copper chelator currently being tested in clinical trials, is used as a comparison.

Methods Breast cell lines, normal, immortalized MCF-10A, premalignant MCF10AT1K.cl2, and malignant MCF10DCIS.com and MDA-MB-231, were treated with CQ or PDTC with or without prior interaction with copper, followed by measurement of proteasome inhibition and cell death. Inhibition of the proteasome was determined by levels of the proteasomal chymotrypsin-like activity and ubiquitinated proteins in protein extracts of the treated cells. Apoptotic cell death was measured by morphological changes, Hoechst staining, and poly(ADP-ribose) polymerase cleavage.

Results When in complex with copper, both CQ and PDTC, but not TM, can inhibit the proteasome chymotrypsin-like activity, block proliferation, and induce apoptotic cell death preferentially in breast cancer cells, less in premalignant breast cells, but are non-toxic to normal/non-transformed breast cells at the concentrations tested. In contrast, CQ, PDTC, TM or copper alone had no effects on any of the cells. Breast premalignant or cancer cells that contain copper at concentrations similar to those found in patients, when treated with just CQ or PDTC alone, but not TM, undergo proteasome inhibition and apoptosis.

Conclusion The feature of breast cancer cells and tissues to accumulate copper can be used as a targeting method for anticancer therapy through treatment with novel compounds such as CQ and PDTC that become active proteasome inhibitors and breast cancer cell killers in the presence of copper.

Introduction

Copper is an essential trace metal for animals. The amount of copper in an organism is tightly regulated [1,2]. Angiogenesis, the growth of a tumor blood supply, is essential for tumor growth, invasion, and metastasis [3-6]. It has been shown that tumors, without a blood supply, do not grow larger than 1 to 2

5,7-DiCl-8-OHQ = 5,7-dichloro-8-hydroxyquinoline; 8-OHQ = 8-hydroxyquinoline; APS = advanced photon source; CQ = clioquinol; DMEM = Dulbecco's modified Eagle medium; DMSO = dimethylsulfoxide; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; PARP = poly(ADP ribose) polymerase; PBS = phosphate buffered saline; PDTC = pyrrolidine dithiocarbamate; TM = tetrathiomolybdate.

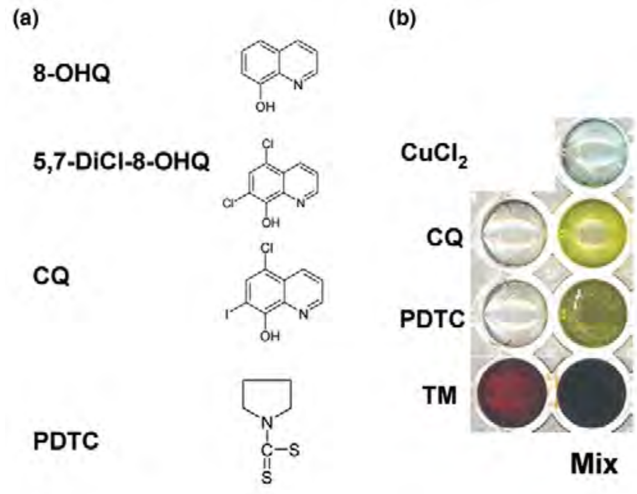
mm³ [7]. Molecular processes of angiogenesis that require copper as an essential cofactor include stimulation of endothelial growth by tumor cytokine production (i.e., vasoendothelial growth factor), degradation of extracellular matrix proteins by metalloproteinases, and migration of endothelial cells mediated by integrins [3-12]. Consistently high levels of copper have been found in many types of human cancers, including breast, prostate, colon, lung, and brain [13-21]. Three anti-copper drugs have been tested in clinical trials [8,9], particularly tetrathiomolybdate (TM), a copper chelator, which was originally used for patients with Wilson's disease [8,11]. TM has been found to be effective in impairing the growth of mammary tumors in HER2/neu transgenic mice [22] and lung metastatic carcinoma in C557BL6/J mice [23]. In a phase I clinical trial with patients suffering from metastatic cancers, TM therapy achieved stable disease in five of six patients who were copper-deficient [11]. However, the disease advanced in some other patients before copper levels were sufficiently lowered [8,9,11]. These reports support the idea of copper control as an anticancer strategy.

Apoptosis, an evolutionarily conserved form of cell death, is the process by which a cell will actively commit suicide under tightly controlled circumstances [24]. Apoptosis occurs in two physiological stages, commitment and execution [25,26]. Activation of effector caspases leads to apoptotic execution probably through the proteolytic cleavage of important cellular proteins [27], such as poly(ADP-ribose) polymerase (PARP) [28], and the retinoblastoma protein [29-31]. Other hallmarks of apoptosis include cellular shrinkage, membrane blebbing, and DNA fragmentation [25-27].

The ubiquitin/proteasome system plays an important role in the degradation of cellular proteins. This proteolytic system involves two distinct steps, ubiquitination and degradation [32,33]. The eukaryotic proteasome contains at least three known activities, which are associated with its β subunits. These are the chymotrypsin-like (cleavage after hydrophobic residues, β5 subunit), trypsin-like (cleavage after basic residues, β2 subunit), and caspase-like or peptidyl-glutamyl peptide-hydrolyzing (cleavage after acidic residues, β1 subunit) activities [34,35]. Inhibition of the proteasomal chymotrypsin-like activity has been found to be associated with induction of apoptosis in tumor cells [36-41].

Most recently, we discovered that several organic-copper (but not zinc or nickel) compounds, such as bis-8-hydroxyquinoline-copper(II), potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* and in human tumor cell culture [42]. Inhibition of the proteasome activity by organic copper compounds occurs very rapidly in tumor cells (15 minutes), followed by induction of apoptosis. Neither proteasome inhibition nor apoptosis were found in human normal or non-transformed cells under the same treatment. Most importantly, proteasome inhibition and apoptosis were also detected in copper-containing tumor cells treated with 8-hydroxyquinoline (8-OHQ; Fig. 1a). None of these events occurred in cells treated with either inorganic copper, ligand-treated cells that did not contain copper, or pretreatment with the closely related nickel followed by addition of the ligand [42]. We also found that 5,7-dichloro-8-hydroxyquinoline (5,7-DiCl-8-OHQ; Fig. 1a) synthesized to contain copper was a potent proteasome inhibitor and apoptosis inducer [42].

Figure 1



Copper complex formation as indicated by color change. **(a)** Chemical structures of compounds referenced in the text: 5,7-DiCl-8-OHQ, 5,7-dichloro-8-hydroxyquinoline; 8-OHQ, 8-hydroxyquinoline; CQ, clioquinol; PDTC, pyrrolidine dithiocarbamate. **(b)** 50 mM of PDTC, CQ, or tetrathiomolybdate (TM) were mixed in a 1:1 molar ratio with $CuCl_2$. All solutions were made in dimethylsulfoxide. In each case the appearance of intensified color indicated formation of a copper complex.

Clioquinol (5-chloro-7-iodo-8-hydroxyquinoline; CQ; Fig. 1a) belongs to the quinoline class of compounds and is structurally similar to 5,7-DiCl-8-OHQ. This class of compounds possesses an established toxicology profile with the US Pharmacopoeia [43]. During the 1950s to the 1970s, CQ was used as an antibiotic [44,45]; however, it was withdrawn due to association with subacute myelo-optic neuropathy possibly due to overdose and/or a reversible vitamin B_{12} deficiency [44,46-48]. Recently, interest in CQ has reemerged due to studies involving its use, in combination with B_{12}, for treatment of Alzheimer's disease [43,49,50]. Regardless of it being a controversial compound, CQ can still serve as a model compound from which analogs could be developed that exploit its copper binding potential but avoid its negative associations. CQ is a lipophilic compound that is capable of forming stable complexes with copper(II) ions [51]. In a phase II clinical trial, CQ, at a starting concentration of 3.3 mg/kg, the same order of magnitude of treatment used in mice, was found to be well-tolerated and suitable for further study [49]. Examination of CQ in animal studies has continued to further characterize its effects [52].

Dithiocarbamates are a class of metal chelating compounds. These compounds have previously been used in the treatment of bacterial and fungal infections, and have been considered for use in the treatment of AIDS [53,54]. Pyrrolidine dithiocarbamate (PDTC; Fig. 1a) is a synthetic antioxidant and inhibitor of NF-κB that is capable of binding copper [55,56]. PDTC and other dithiocarbamates have been found to induce apoptosis in conjunction with copper in different types of cancer cells [55,57]. Previously we found a synthetic PDTC containing copper was a potent proteasome inhibitor and apoptosis inducer [42].

Here we show that CQ and PDTC are capable of binding copper, spontaneously forming new complexes that have proteasome-inhibitory and apoptosis-inducing activities to cancer but not normal/non-transformed breast cells, and that premalignant or cancer breast cells cultured to contain elevated copper are sensitive to treatment with CQ or PDTC alone. In contrast, TM-copper or TM alone had no effects in the same experiments. We propose that targeting highly elevated copper can be tumor-specific and that formation of an active anticancer proteasome inhibitory complex between CQ or PDTC and tumor cellular copper is a novel strategy that has great potential for breast cancer therapies.

Materials and methods

Chemicals and reagents

CQ, PDTC, disulfiram (tetraethyl thiuram disulfide), tetramethyl thiuram disulfide, methyl propyl disulfide, allyl disulfide, isopropyl disulfide, TM, $CuCl_2$, dimethylsulfoxide (DMSO), bisbenzimide Hoechst No. 33258 stain, cholera toxin, hydrocortisone, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), epidermal growth factor, and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). F12 medium, DMEM, horse serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA, USA). Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibody to human PARP was from Roche Applied Science (Indianapolis, IN, USA). Mouse monoclonal antibody to human ubiquitin was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA).

Cell culture and lysates preparation

MCF10A (normal-MCF10), MCF10AT1K.cl2 (premalignant-MCF10), and MCF10dcis.com (malignant-MCF10) cells were cultured as described previously [58]. Briefly, normal-MCF10 and premalignant-MCF10 cells were cultured in 1:1 F12/DMEM prepared as follows: 500 ml of media was supplemented with 5.26% (v/v) horse serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 52.55 μg of cholera endotoxin, 5 mg insulin, 10 ml of 1 M $NaHCO_3$, 10 μg of epidermal growth factor, and 250 μg hydrocortisone. Malignant-MCF10 cells were cultured in 1:1 F12/DMEM media supplemented with 5.26% (v/v) horse serum, 10 ml of 1 M $NaHCO_3$, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM media containing 10% (v/v) fetal bovine serum and 100 units/ml of penicillin, 100 μg/ml of streptomycin. All cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO_2. For copper enrichment experiments, premalignant-MCF10 or MDA-MB-231 cells were cultured in media further supplemented with 25 μM $CuCl_2$ for 3 days to 2 weeks. Whole cell extracts were prepared as described previously [29]. Briefly, cells were harvested, washed with PBS twice, and homogenized in a lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP40 (v/v), 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) for 30 min at 4°C. Afterwards, the lysates were centrifuged at 12,000 g for 30 minutes, and the supernatants were collected as whole cell extracts.

Color change and precipitate formation reactions

CQ, PDTC, TM, and $CuCl_2$ were dissolved in DMSO to a final concentration of 50 mM. Then $CuCl_2$ was mixed with each in a 1:1 ratio and qualitatively examined for color change and precipitate formation. After mixing, solutions were heated and vortexed repeatedly until clear. For the visual studies, solutions were examined for color change and precipitation as indicators of complex formation. In cellular studies, however, stock concentrations were kept lower (10 and 20 mM) prior to dilution during mixing in order to prevent precipitation.

Cell proliferation assay

The MTT assay was used to determine the effects of these agents on overall proliferation of cells. Cells were plated in a 96-well plate and grown to 70–80% confluency, followed by addition of each compound at an indicated concentration for 24 h. MTT (1 mg/ml) in PBS was then added to wells and incubated at 37°C for 4 h to allow for complete cleavage of the tetrazolium salt by metabolically active cells. Next, MTT was removed and 100 μl of DMSO was added, followed by colorimetric analysis using a multilabel plate reader at 560 nm (Victor^3; PerkinElmer (Wellesley, MA, USA)). Absorbance values plotted are the mean from triplicate experiments.

Cellular and nuclear morphology analysis

A Zeiss (Thornwood, NY, USA) Axiovert 25 microscope was used for all microscopic imaging with either phase contrast for cellular morphology or fluorescence for nuclear morphology with Hoechst staining. For fluorescent nuclear morphology analysis, Hoechst stain was used as follows. Cells, either attached in plates or collected as a detached fraction, were washed once with ice cold PBS. Cells were then fixed in ethanol for 1 h and afterwards washed with ice cold PBS. Cells were stained with 50 μM Hoechst and kept in the dark at 4°C for 30 minutes and then visualized using fluorescence microscopy. Punctate and bright staining, or granular and bright staining nuclei were considered apoptotic.

Copper pretreatment and ligand post-treatment

To simulate the *in vivo* copper status of cancer cells, premalignant-MCF10 and MDA-MB-231 cells were cultured in media containing 25 μM copper as done previously with prostate PC-3 cells [42]. MDA-MB-231 cells were cultured for a minimum of 48 h and premalignant-MCF10 cells were cultured for a minimum of 2 weeks. After copper enrichment culturing, cells were washed with PBS and then treated for the indicated hours using standard cell media containing TM (25 μM), CQ (1 to 100 μM), or PDTC (1 or 10 μM).

Cellular copper measurement

Premalignant-MCF10 cells were cultured for 2 weeks in culture media with or without 25 μM $CuCl_2$. Cells were collected and counted to determine total cells in the sample. Samples were spun down, washed with PBS, and provided to Quantum Labs (Wixom, MI, USA) for graphite furnace analysis to determine total copper in each sample.

Western blot analysis

Cells were treated as indicated (see Figure legends). Afterwards, cells were harvested and lysed. Cell lysates (50 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane, followed by visualization using the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Western blot analysis was performed using specific antibodies to ubiquitin and PARP as described previously [36]. Proteasome inhibition was measured as accumulation of ubiquitinated proteins and apoptosis by cleavage of PARP [36].

Analysis of the proteasome chymotrypsin-like activity in whole cell extracts

Whole cell extracts (10 μg) of cells treated as indicated were incubated for 60 minutes at 37°C in 100 μl of assay buffer (50 mM Tris-HCL, pH 7.5) with 40 μM of fluorogenic substrate for the proteasomal chymotrypsin-like activity. After incubation, production of hydrolyzed 7-amino-4-methylcoumarin (AMC) groups was measured using a Victor 3 Multilabel Counter with an excitation filter of 380 nm and an emission filter of 460 nm (PerkinElmer, Boston, MA, USA). Changes in fluorescence were calculated against non-treated controls and plotted with statistical analysis using Microsoft Excel™ software.

Results

CQ and PDTC spontaneously react with copper to form a new complex

In order to use endogenous elevated tumor copper as a targeting mechanism for breast cancer therapy (Fig. 2), it is necessary that the ligand under consideration be capable of reacting spontaneously with copper to form a new complex. Complex formation reactions, particularly those involving metal, can result in dramatic color changes and/or precipitate formation. To test the reactivity of CQ and PDTC with copper, 50 mM of each was added to a 50 mM solution of copper (II) chloride (Fig. 1). The reaction of CQ and PDTC with copper, in DMSO, results in a dramatic color change (Fig. 1), indicating a chemical reaction has occurred and a metal complex has formed. These results are consistent with previous publications showing that both CQ and PDTC are strong copper chelators [51,55]. Therefore, these ligands may be capable of combining with endogenous tumor copper and forming a reactive complex.

Figure 2

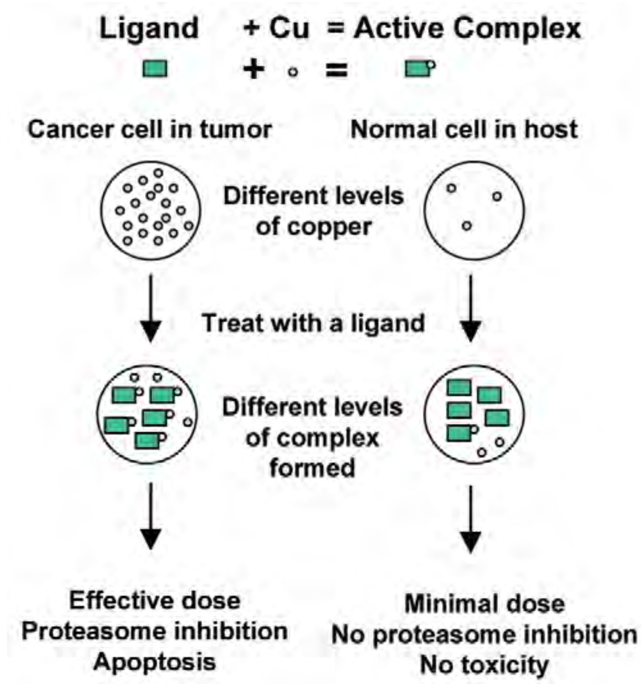


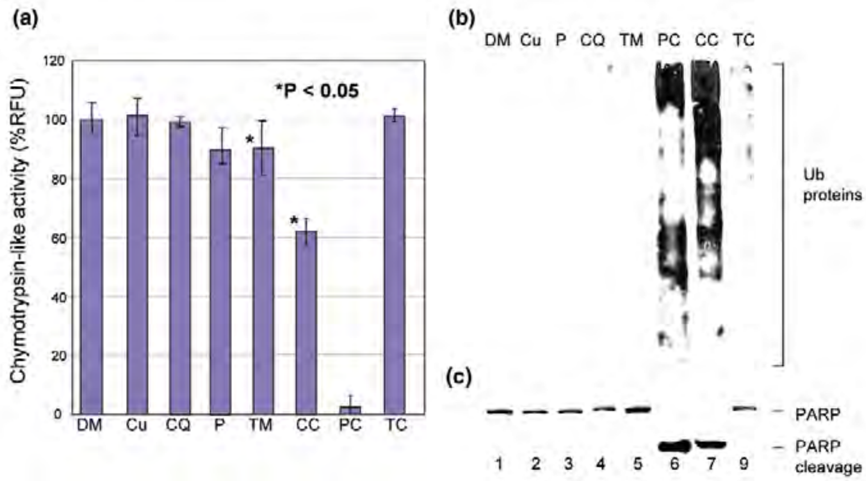
Diagram of a proposed copper-targeting therapeutic strategy. Cancer cells contain high levels of copper compared to normal cells. Upon treatment with a copper-binding ligand, a proteasome inhibiting copper complex will be formed. Only a minimal amount of complex should be formed in normal cells, therefore making them resistant to proteasome inhibition. In contrast, cancer cells may have a high dose of complex formed and are thus sensitive to proteasome inhibition, resulting in apoptosis. Copper forms the basis of the selection criteria between normal and tumor cells.

The CQ-copper mixture has been further examined by the advanced photon source (APS) of Argonne National Laboratories (Argonne, IL, USA). The result is consistent with formation of a new complex between CQ and copper in solution (unpublished data). Furthermore, samples of a PDTC-copper mixture will be analyzed by the APS to confirm complex formation and the resulting structure. The details of these studies will be presented in a future manuscript.

CQ and PDTC combine with copper to form proteasome-inhibitory complexes

As both compounds can form a complex with copper, as indicated by color change (Fig. 1), we then tested whether these

Figure 3



Inhibition of proteasome activity and apoptosis induction in MDA-MB-231 breast cancer cells by clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper. MDA-MB-231 breast cancer cells were treated with 20 μM copper (Cu), CQ, CQ + copper (CC), tetrathiomolybdate (TM), TM + copper (TC), or 10 μM PDTC (P), or PDTC + copper (PC), using DMSO (DM) as a control. Cells were collected after 24 h treatment and analyzed for proteasome inhibition. **(a)** Proteasome activity as measured in released fluorescence units (RFUs) by release of 7-amino-4-methylcoumarins (AMCs) from substrate specific for chymotrypsin-like activity. **(b)** Western analysis for accumulation of ubiquitinated proteins as an indicator of proteasome inhibition. Treatment with PDTC + copper (PC; 10 μM) or CQ + copper (CC; 20 μM) results in reduced release of AMCs and ubiquitinated protein accumulation, suggesting proteasome inhibition. **(c)** Western analysis for cleavage of poly(ADP ribose) polymerase (PARP) as an indication of apoptosis. Treatment with CQ + copper (20 μM) or PDTC + copper (10 μM) results in cleavage of PARP, indicating that these complexes are capable of inducing apoptosis.

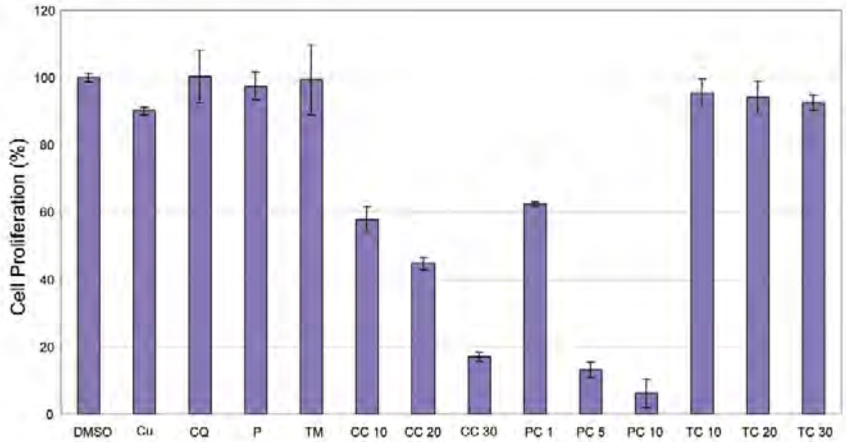
complexes were capable of inhibiting the proteasome activity in intact cells. Breast cancer MDA-MB-231 cells were treated with copper, CQ, CQ-copper mixture, PDTC, or PDTC-copper mixture, using TM and TM-copper mixture as controls. After a 24 h treatment, cells were collected and the cell extracts were prepared for analysis of proteasome inhibition by the chymotrypsin-like activity assay (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). We found that both CQ-copper and PDTC-copper mixtures significantly inhibited the proteasome activity in MDA-MB-231 cells, as indicated by decreased levels of the proteasomal chymotrypsin-like activity (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). The PDTC-copper mixture is more potent than that of CQ-copper (Fig. 3a). Copper, CQ, or PDTC alone had no effect. Interestingly, we found that TM and the TM-copper mixture had little to no proteasome-inhibitory activity (Fig. 3), supporting the inactive complex nature of TM-copper [42]. These data support that CQ and PDTC can combine spontaneously with copper to form a proteasome-inhibitory complex.

Although we have shown that copper alone can inhibit the activity of a purified proteasome [42], it is still possible that dithiocarbamates could be oxidized by copper to thiuram disulfides [59], which could be responsible for the observed proteasome inhibition. We therefore tested the effects of two thiuram disulfides and three disulfides on the proteasome activity. In the absence of copper, disulfiram (tetraethyl thiuram disulfide) and tetramethyl thiuram disulfide are incapable of inhibiting the proteasomal activity of MDA-MB-231 cell extract at micro-molar concentrations (data not shown). In addition, none of the tested disulfides, methyl propyl disulfide, allyl disulfide, and isopropyl disulfide, could inhibit the proteasome activity under the cell-free conditions (data not shown). This result suggests that complex formation between PDTC and copper, rather than general oxidation of PDTC to thiuram disulfide, is the likely mechanism of proteasome inhibition. Furthermore, we have found and reported that production of H_2O_2 does not occur in this system and that reductants do not block copper-mediated proteasome-inhibitory activity, supporting the idea that mechanisms other than oxidation are involved in proteasome inhibition [42]. This suggests that general oxidation or oxidation of dithiocarbamates is not sufficient to result in proteasome inhibition at these concentrations in these systems.

CQ and PDTC when mixed with copper block proliferation of breast cancer MDA-MB-231 in a dose-dependent manner

After finding that CQ-copper and PDTC-copper mixtures could inhibit proteasome activity (Fig. 3a,b), we measured the effects of each compound on breast cancer cell proliferation (Fig. 4). We found that, associated with proteasome inhibition, the CQ-copper and PDTC-copper complexes inhibited cellular proliferation in a dose-dependent manner. CQ-copper showed 40% inhibition at 10 μM and increased to approximately 80% inhibition at 30 μM (Fig. 4). The PDTC-copper mixture inhibited proliferation by 40% at 1 μM and greater than 90% inhibition at 10 μM (Fig. 4). In contrast, copper, CQ,

Figure 4



Anti-proliferative effects of clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper. MDA-MB-231 cells were treated for 24 h with the following: copper (Cu; at 30 μM); CQ (CQ; at 30 μM); PDTC (P; 10 μM); tetrathiomolybdate (TM; 30 μM); CQ + copper (CC; 10, 20, 30 μM); PDTC + copper (PC; 1, 5, 10 μM); TM + copper (TC; 10, 20, 30 μM); or dimethylsulfoxide (DMSO) as a control. After 24 h the media was removed and cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) solution. Measurement of MTT conversion by absorbance at 560 nm showed that MDA-MB-231 cells responded in a dose-dependent manner to CQ-copper and PDTC-copper complexes. This suggests that these organic-copper complexes act as anti-proliferative agents.

PDTC, or TM alone or TM mixed with copper had no significant effect (Fig. 4). The ranking of these compounds with respect to their ability to inhibit breast cancer cell proliferation matches well with their ability to inhibit the cellular proteasome activity (Figs. 4 versus 3a). Due to the nature of the MTT assay and the inability to separate apoptosis from growth arrest, both possible outcomes of the proteasome inhibition, IC_{50} values of these complexes were not measured. These data suggest that CQ and PDTC can spontaneously combine with copper to form an anti-proliferative complex.

CQ and PDTC combine with copper to form a product toxic to malignant-MCF10 and MDA-MB-231 and premalignant-MCF10 cells, but non-toxic to normal-MCF10 breast cells

We found that the same CQ-copper and PDTC-copper complexes capable of proteasome inhibition (Fig. 3a, b) also demonstrated apoptosis induction, as shown by cleavage of PARP (Fig. 3c). In the absence of copper, neither CQ nor PDTC was able to induce apoptosis at these concentrations (Fig. 3c, lanes 6 and 7 versus lanes 3 and 4). TM, in the presence or absence of copper, does not induce apoptosis, further supporting TM's action as passive chelation and elimination of copper. These data support the idea that CQ and PDTC, but not TM, can combine spontaneously with copper to form a proteasome-inhibitory and apoptosis-inducing complex.

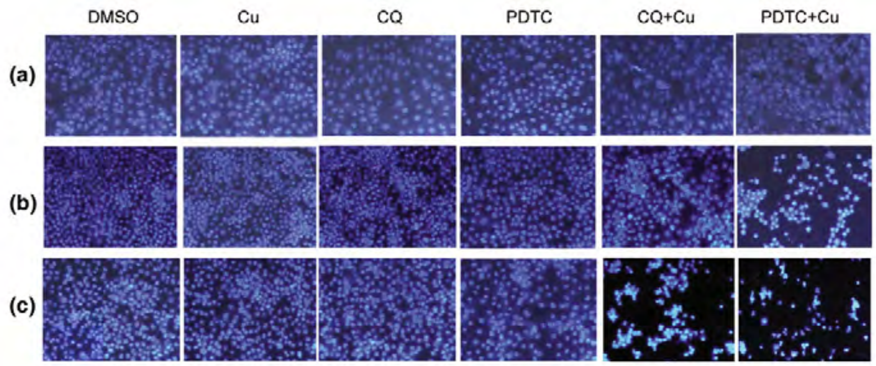
To determine whether the CQ-copper and PDTC-copper complexes have differential effects on normal and tumor breast cells, the MCF10 series of cells [58] were then treated with CQ alone, copper alone, or the product of a 1:1 mixture of each at 20 μM (Fig. 5). The 20 μM CQ-copper complex induces apoptotic nuclei within 24 h for both premalignant- and malignant-MCF10 cells (10% and 65%, respectively; Fig. 5b, c). The malignant-MCF10 cells fully detached, suggesting that these cells were more sensitive to the complex than the premalignant cells. However, the normal-MCF10 cells demonstrated no apoptotic nuclei after 24 h of treatment with the CQ-copper complex (<2%; Fig. 5a).

We then tested the effects of the PDTC-copper mixture. The three breast cell lines were treated with PDTC alone, copper alone, or their mixture at 5 μM for 24 h. Again, both the premalignant- (63%; Fig. 5b) and the malignant-MCF10 (75%; Fig. 5c) cells showed a dramatic induction of apoptotic nuclei after treatment with the mixture, while the normal-MCF10 cells (<2%; Fig. 5a) showed no apoptosis induction from the mixture. As a control, neither CQ alone, PDTC alone, nor copper alone had effect on any of these cell lines (<2% in all the cases; Fig. 5). These data suggest that CQ and PDTC can spontaneously bind with copper and that the resulting complex is an apoptosis inducer to premalignant and cancerous, but not normal/non-transformed, breast cells, suggesting that such a complex if formed in a normal cell would not be toxic, but would be toxic in tumor cells.

CQ and PDTC in complex with copper do not inhibit proteasome activity in normal breast MCF10A cells

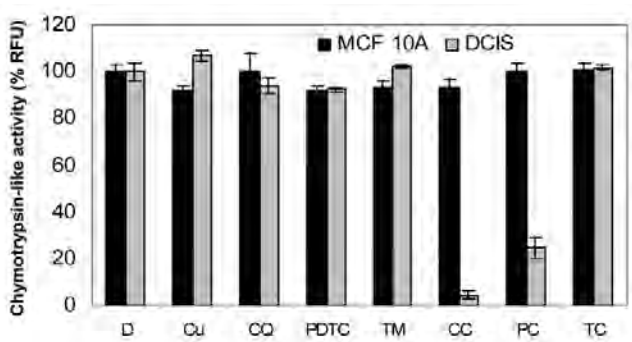
To better understand the mechanism of resistance in normal breast cells to apoptosis induction by these organic-copper complexes, we treated both normal- and malignant-MCF10 cells with CQ-copper and PDTC-copper complexes and measured changes in the proteasome activity levels. Both cell lines were treated with 20 μM Cu, CQ, TM, CQ-copper, and

Figure 5



Induction of apoptosis by clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper complexes in premalignant- and malignant-MCF10 cells. CQ and PDTC were mixed in a 1:1 molar ratio with $CuCl_2$. Normal-, premalignant, and malignant-MCF10 cells were treated with CQ alone (CQ), copper alone (Cu), or CQ + copper (CQ+Cu) at 20 μM, or PDTC alone or PDTC + copper (PDTC+Cu) at 5 μM for 24 h. Dimethylsulfoxide (DMSO) was used as a control. After treatment, cells were stained with Hoescht for determination of apoptotic nuclei. Nuclei that were punctate or granular and bright were considered apoptotic. **(a)** The normal MCF10 cell line showed no apoptosis induction from the ligand, copper, or mixture (<2% in all cases). However, both **(b)** the premalignant MCF10AT1K.cl2 and **(c)** the malignant MCF10DCIS.com cells showed a dramatic induction of apoptotic nuclei after treatment with the mixture. **(b)** The percentages of apoptosic nuclei in MCF10AT1K.cl2 cells were: CQ+Cu, 10%; PDTC+Cu, 63%; and others <2%. **(c)** The percentages of apoptotic nuclei in MCF10DCIS.com cells were: CQ+Cu, 65%; PDTC+Cu, 75%; and others <2%.

Figure 6



Clioquinol (CQ) + copper and pyrrolidine dithiocarbamate (PDTC) + copper fail to inhibit proteasome activity in normal MCF 10 (MCF-10A) cells. Normal MCF-10A and malignant mCF-10 (DCIS) breast cells were treated with 20 μM of copper (Cu), CQ, CQ + copper (CC), tetrathiomolybdate (TM), TM + copper (TC), or 5 μM PDTC or PDTC + copper (PC). Dimethylsulfoxide (D) was used as a control. After 24 h, cells were collected and lysed. Lysates were analyzed for chymotrypsin-like activity and showed as released fluorescence units (RFUs). The complexes were capable of eliminating proteasome activity in DCIS cells but not in MCF-10A cells. This strongly suggests that these metal complexes do not inhibit proteasome activity in normal breast cells.

TM-copper, or 5 μM PDTC and PDTC-copper (Fig. 6). We found that PDTC-copper and CQ-copper both strongly inhibited proteasome activity in malignant but not in normal cells (Fig. 6). Again, TM or the TM-copper mixture had no effects on either of the cell lines (Fig. 6). These data suggest that these organic-copper complexes do not inhibit the proteasome and, therefore, do not induce apoptosis in normal breast cells, further protecting normal cells from toxicity.

Premalignant-MCF10 cells accumulate copper when cultured in copper-enriched conditions

A difficulty with examining the effectiveness of copper targeting in cell culture models is that cultured cancer cells seem to possess low to trace levels of copper [42]. This differs from the *in vivo* situation where cancer cells and tissues can contain micromolar concentrations of copper. In one study, the serum copper in breast cancer patients was approximately 2 μg/100 ml (equivalent to 0.3 μM) [60], while another study showed that the plasma copper levels in the malignant prostates were 124 μg/100 ml (equivalent to 19.5 μM) [16].

To simulate the *in vivo* situation, premalignant-MCF10 breast cells were cultured in media enriched with 25 μM $CuCl_2$ for at least 2 weeks (see Materials and methods). Afterwards, cells were collected and subject to graphite furnace analysis to determine copper content (Table 1). The results of the analysis show that these cells can accumulate at least 16 times more copper when cultured in copper-enriched media (referred to here as copper-enriched cells) than when in a normal culture and an individual enriched cell has at least an order of magnitude more copper than a standard culture cell. Given a volume of 10 ml, this would be equivalent to 6 μM. Previously, we pretreated prostate cancer PC-3 cells with 100 μM $CuCl_2$ for 48 h, which resulted in cellular copper levels being increased to 0.2 μM [42]. These data show that, in our enrichment system, premalignant-MCF10 cells can accumulate similar copper concentrations to those found in patients.

Table 1

Accumulation of copper by MCF10AT1K.cl2 cells

Cell line	No. of cells in sample	Total copper (μg)	Copper per cell
KCL2	5,725,000	ND	(1.75×10^{-8})[a]
KCL2-Cu25	1,468,750	0.4	2.72×10^{-7}

[a]This is theoretical content assuming no more than 0.1 μg total copper, which is the minimal detection limit. Because cells grown in media without copper enrichment had no detectable copper, the number reported is the highest possible content per cell based on the minimum detection. ND, not detected.

Copper-enriched breast pre-malignant and cancer cells are sensitive to treatment with CQ or PDTC alone

Fundamental to the strategy we are presenting is the ability of a normally non-toxic ligand to bind with endogenous tumor cellular copper (Fig. 2). Studies in various cancer cells and tissues have found that patients can have copper concentrations in the micromolar ranges in those tissues [16,60]. Similarly, when premalignant-MCF10A cells are cultured in copper they can contain micromolar concentrations of copper (Table 1). We therefore tested the effects of CQ or PDTC alone in copper-enriched breast premalignant or cancer cells.

We first treated the copper-enriched premalignant-MCF10 cells with CQ alone. CQ at 1 to 10 μM caused apoptotic morphological changes of these copper-containing cells (Fig. 7a). Consistent with the morphology study, after 24 h treatment with CQ, these copper-enriched cells underwent extensive apoptosis, measured by the appearance of the PARP cleavage fragment (Fig. 7c). In contrast, premalignant-MCF10 cells that did not contain elevated copper were highly resistant to 25 μM of CQ (Fig. 7c, lanes 1 and 2). Similarly, copper-enriched premalignant-MCF10 cells were also sensitive to treatment with PDTC, but not TM (data not shown; Fig. 7b, d, e).

We also found that copper-enriched breast cancer MDA-MB-231 cells adopt apoptotic morphology after post-treatment with CQ or PDTC, but not TM (Fig. 7b). In the same experiment, lysates of these cells were subjected to western analysis. Both CQ and PDTC were capable of inducing proteasome inhibition and apoptosis in copper-pretreated MDA-MB-231 cells, as measured by accumulation of ubiquitinated proteins and cleavage of PARP, respectively (Fig. 7d, e). This is dramatically different from the behavior of these compounds in the absence of copper or in non-copper enriched cells (Fig. 7d, e versus Fig. 3b, c). In contrast, TM neither inhibited the proteasome activity nor induced apoptosis in these copper-enriched cells (Fig. 7b, d, e). These data support the idea that CQ and PDTC can spontaneously bind with copper in copper-enriched breast cancer cells and form an apoptosis-inducing complex and that cells containing trace or undetectable amounts of copper are resistant to this effect. It is possible, therefore, that CQ and PDTC act as apoptosis inducers through proteasome inhibition in a copper-dependent manner and can do so in cancer cells that contain copper in concentrations similar to those found in patients' tissues and serum.

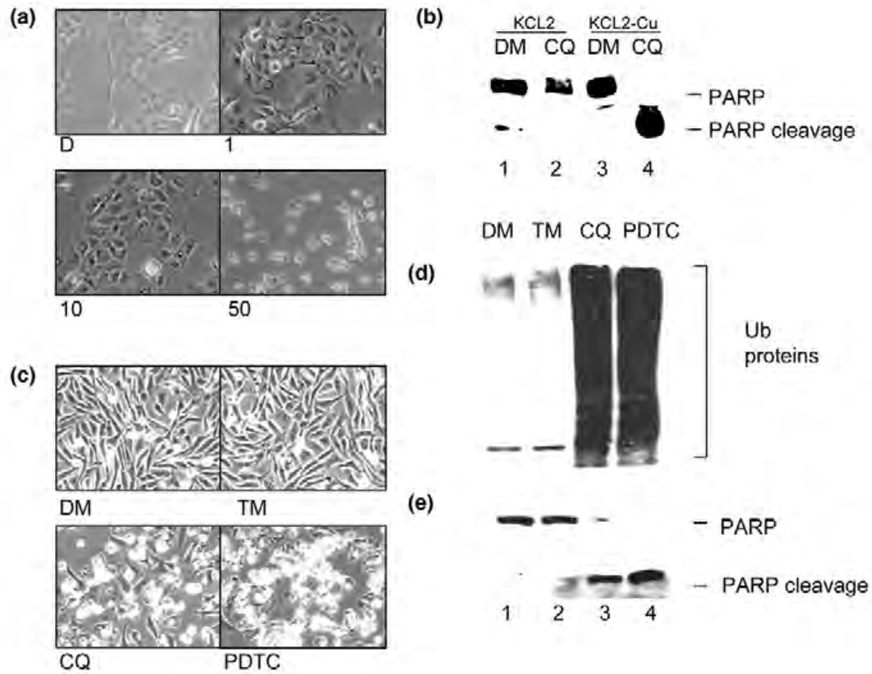
Discussion

A difficulty facing most cancer chemotherapy is the inability to discriminate between normal and malignant cells. Anti-angiogenesis and proteasome inhibition may be effective approaches to cancer therapy due to the dependence of cancer on these activities [9,37,61]. Unique among the trace metals, copper is required for angiogenesis [8-11]. Furthermore, it is well documented that cancer cells and tissues accumulate high concentrations of copper [13,18,21,62-64]. We previously reported that certain types of organic-copper complexes are capable of proteasome inhibition that is not a result of oxidative effects [42]. Therefore, the capability of organic copper to inhibit the proteasome, the necessity of copper for angiogenesis, and the accumulation of copper by cancer cells and tissues allows for a novel therapeutic strategy focusing on elevated copper as a selection mechanism against cancer cells and tissues (Fig. 2).

Our previous study [42] Additional file: 1 only briefly looked at an isolated system and examined the phenomenon of organic ligands binding to copper to form proteasome inhibitors and apoptosis inducers. The current study confirms and significantly expands our original findings. Specifically, this study examines a complete breast cancer system, including normal, premalignant and malignant cells. Furthermore, this study examines compounds that have clinical relevance and expands the copper enrichment studies. Several different approaches have been used in the analysis.

CQ and PDTC are two copper-binding compounds [51,53]. CQ has been investigated for use in Alzheimer's disease in regards to its ability to bind to copper found in amyloid plaques [43,47-50]. PDTC is a synthetic copper-binding antioxidant that has been studied for use in AIDS [53,54]. Previously, we have seen that 5,7-DiCl-8-OHQ (an analog of CQ) and PDTC when in complex with copper possessed strong proteasome-inhibitory and apoptosis-inducing abilities [42]. We report here the ability of CQ and PDTC to spontaneously react with copper, and inhibit the proteasome, which is followed by apoptosis, in breast cancer but not normal cells.

Figure 7



Induction of apoptosis in premalignant-MCF10 breast cells and proteasome inhibition and apoptosis induction in breast cancer MDA-MB-231 cells cultured to contain elevated copper and post-treated with clioquinol (CQ) and pyrrolidine dithiocarbamate (PDTC). **(a,c)** Premalignant-MCF10 (KCL2) cells were cultured in media containing 25 μM copper for two weeks. **(b,d,e)** MDA-MB-231 cells were cultured in 25 μM copper for 48 h. After culturing, cells were washed with PBS and treated with media containing: **(a)** 1, 10, or 50 μM CQ; **(b-e)** 25 μM CQ; 25 μM tetrathiomolybdate (TM); or **(b,d,e)** 10 μM PDTC. An equivalent volume of DMSO (DM) was used as control. KCL2 and MDA-MB-231 cells were examined for **(a,b)** apoptotic morphology and **(c,e)** PARP cleavage. **(d)** MDA-MB-231 cells were also examined by western blot for accumulation of ubiquitinated proteins. **(a,c)** KCL2 cells containing clinically relevant levels of copper were sensitive to treatment by CQ alone, which induced apoptosis. **(c)** KCL2 cells cultured under standard conditions were resistant to treatment by 25 μM CQ. Similarly, MDA-MB-231 cells cultured to contain elevated copper were sensitive to CQ or PDTC and underwent proteasome inhibition as measured by accumulation of **(d)** ubiquitinated (Ub) proteins and apoptosis as evidenced by morphology and **(b,e)** poly (ADP ribose) polymerase (PARP) cleavage. These data suggest that KCL2 and MDA-MB-231 cells cultured to contain clinically relevant levels of copper are sensitive to treatment with CQ or PDTC alone, but not TM.

Our strategy revolves around the idea that a normally inactive or nontoxic organic ligand could bind with copper found in tumor tissues, resulting in a complex capable of proteasome inhibition. It has been shown that cancer cells are more sensitive to proteasome inhibition than normal cells [37,61,65-67]. To that end, we first verified that these two ligands directly interact with copper and form a new metal complex as indicated by dramatic color change (Fig. 1).

Once we verified that these two compounds spontaneously bind with copper and form a new complex we tested these complexes in MDA-MB-231 breast cancer cells to determine whether or not these complexes were proteasome inhibitors. We examined both cellular proteasome activity (Fig. 3a) and accumulation of ubiquitinated proteins (Fig. 3b). We found that treatment with ligand-copper mixtures significantly reduced chymotrypsin-like activity (Fig. 3a) and resulted in accumulation of ubiquitinated proteins (Fig. 3b), indicating proteasome inhibition had occurred. In contrast, ligand alone, copper alone, or TM mixed with copper did not inhibit the proteasome (Fig. 3). Previously we found that copper-mediated accumulation of ubiquitinated proteins is transient [42]. Therefore, the ubiquitinated protein pattern induced by CQ-copper and PDTC-copper shown in Fig. 3b should be considered transient and relevant only to the time point under consideration.

After determining that these organic-copper complexes could inhibit proliferation in MDA-MB-231 cells (Fig. 4), we examined the apoptosis-inducing abilities of the complexes. The organic-copper complexes were capable of inducing apoptosis strongly in malignant-MCF10 and MDA-MB-231, moderately in premalignant-MCF10, and did not induce apoptosis in normal-MCF10 cells (Figs. 5 and 3c). As a control, CQ, PDTC, TM or copper alone, or TM mixed with copper, were incapable of inducing apoptosis (Figs 5 and 3c). Therefore, the primary concerns of the strategy presented were fulfilled: the compound alone shows no toxic effects, the compound when mixed with copper becomes toxic, and the toxicity is limited to cancer cells and is associated with proteasome inhibition.

As these complexes have minimal to no effect on our normal cell line and seem to inhibit tumor cellular proteasome activity, we surmise that their toxicity to cancer cells stems from their proteasome inhibitory activity, to which normal cells are resistant. This was verified by examining proteasome activity in breast normal MCF10 cells compared to malignant-MCF10 cells (Fig. 6). We found that normal-MCF 10 cells did not suffer proteasome inhibition when treated with CQ or PDTC in complex with copper, although the concentrations tested inhibited the proteasome activity in malignant-MCF10 cells (Fig. 6), further supporting the argument that these complexes may be non-toxic to normal cells but are toxic to cancer cells through the mechanism of tumor-specific proteasome inhibition.

In a living organism, cancer cells and tissues accumulate high concentrations of copper [13,18,21,62-64]. To simulate this *in vivo* situation, premalignant-MCF10 and cancer MDA-MB-231 breast cells were cultured in copper-enriched media for either 2 weeks (premalignant-MCF10) or 72 h (MDA-MB-231). Afterwards, premalignant-MCF10 cells were collected and subjected to graphite furnace analysis to determine copper content. We found that premalignant-MCF10 cells were capable of accumulating concentrations of copper similar to those found in patient tissues (Table 1) and contained at least 16 fold more copper than cells cultured in standard media.

Once we had established cultures of premalignant-MCF10 cells enriched with copper, we then treated those cells with CQ or PDTC alone. Both CQ (25 μM) and PDTC (1 μM) induced apoptosis after treatment (Fig. 7a, c; data not shown). In cells cultured in enriched copper conditions, the compounds at similar concentrations had no effect (Figs 5 and 3c). Similarly, the breast cancer cell line MDA-MB-231, when cultured in elevated copper, is sensitized to apoptosis induction associated with proteasome inhibition with CQ and PDTC alone (Fig. 7b, d, e). This further supports our proposal that the compounds studied can use the increased copper load in cancer cells to form a proteasome inhibitor and an apoptosis inducer, whereas in the absence of this copper load these compounds have minimal to no effect at these concentrations.

The data presented here supports the novel concept of using accumulated copper in breast cancer cells and tissues as a selection method for chemotherapy. Non-toxic organic compounds such as CQ or PDTC can spontaneously bind with copper and form a proteasome inhibitor and an apoptosis inducer that has no effect on normal cells. Cancer cells, containing elevated copper, are sensitive to treatment with these organic compounds. Normal cells, containing trace amounts of copper, are resistant to these effects (Fig. 2). Both CQ and PDTC have been previously explored for use in other diseases and we believe these data support further investigation of these and other similar compounds in an anticopper/anticancer strategy. Most recently, another group also reported the anticancer activity of CQ [52]. Our data presented here may have provided a mechanistic interpretation for their findings.

The exact mechanisms of the copper-ligand combination are unclear at this time. However, it is apparent that cells cultured to contain elevated copper become sensitive to treatment with the ligands alone. We have future plans to work with the APS at Argonne National Laboratory to determine the final state of the ligand-copper complexes in cells. This should assist in further understanding why copper-enriched cells are sensitive to treatment with ligands that bind copper to form proteasome-inhibiting complexes. It should be noted that the system we have presented in this report is limited by looking at immortalized breast cancer cells rather than true normal primary cell lines. Future experiments should examine not only normal primary lymphocytes in culture but also animal studies to further confirm the effect on normal cells and tissues. Additional studies on cells that naturally contain elevated copper such as kidney, liver, and hematopoietic cells are also warranted.

Conclusion

A unique feature of cancer cells is to accumulate high concentrations of copper [13,18,21,62-64]. We believe a potential strategy for cancer chemotherapy could involve the use of organic ligands that act as copper sensors and bind with the elevated copper in cancer cells and tissues. These complexes would act as proteasome inhibitors and apoptosis inducers to tumor cells. Because normal cells contain only trace amounts of copper, the organic ligands should form far fewer complexes with copper in them, thus exposing the normal cells to a minimal dose and reducing toxicity. We propose that treatment with copper-binding compounds such as CQ and PDTC will result in these compounds behaving as tumor 'sensors' using copper as a selection criterion. Therefore, this approach may convert the proangiogenic co-factor copper into a cancer-specific killing agent.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KD and DC contributed equally to this manuscript. KD participated in the design of the study, data collection and interpretation, and manuscript preparation. DC participated in study design, data collection and interpretation, and manuscript preparation. SO and QCC participated in data collection. FRM participated in the design of the study, data interpretation, and manuscript preparation. QPD was responsible for the design of the study, data interpretation, and manuscript preparation as well as supervision of this project. All authors have read and approved the final manuscript.

Additional files

The following Additional files are available online:

Additional File 1

A PDF file of reference [42].
See http://www.biomedcentral.com/content/supplementary/bcr1322-S1.pdf

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Apoptotic-inducing activity of novel polycyclic aromatic compounds in human leukemic cells

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Abstract. Persistent but relatively limited research has been devoted to the use of compounds related to polycyclic aromatic hydrocarbons (PAH) as anticancer agents. In previous reports, we have described the cytotoxicity of a number of new and novel PAH against human cancer cell lines. However, the involved molecular mechanisms of inducing cell death were not elucidated. In the current study, we describe the apoptotic pathway as apparently playing a crucial role in induced cell death in human leukemia Jurkat T cells by several diamide and diamine PAH that contain chrysene as their core aromatic ring system. Structure-activity relationships were analyzed. Importantly, no effect was demonstrated in a normal, non-transformed line of human natural killer cells. These results provide additional evidence for the potential chemotherapeutic use of PAH.

Introduction

Persistent but relatively limited research has been focused on the use of PAH-compounds as anticancer agents. For example, Bair *et al* (1,2) have studied a number of unique compounds of this type and have reported a close correlation between antitumor activity and the shape of their polyaromatic system. They have developed several, highly active benzylic aminopropanediols using structure-activity relationships (SAR) as the basis for synthesizing new derivatives. These compounds were believed to interact with DNA by intercalation and to act as topoisomerase inhibitors. However, they were not able to produce a definitive correlation between the ability of these compounds to bind to DNA and the resultant cytotoxic activity (3,4). Clinical studies of two of the most active of these napththalimides failed as a result of CNS toxicity. To improve the potency and the toxicity:benefit ratio, naphthalene was changed to anthracene to serve as the chromophore. While the resulting analog, azonafide, showed enhanced potency, and had many of the characteristics similar to those of other classes of DNA intercalators, it did not definitively localize in the nucleus (5,6). Other differences from existing DNA intercalating agents such as mitoxantrone (7) include the failure of inhibition of topoisomerase II at equicytotoxic concentrations. Denny *et al* have published a number of crucial studies that raise important questions as to the exact target of a number of related compounds (8).

In the current study, we examined the apoptosis-inducing activity of several PAH that contain chrysene as the core aromatic ring system (9,10). Previous examination of SAR utilizing their cytotoxicity against a number of human cancer cell lines (9-11) revealed interesting correlations of activity with modification of types and numbers of functional groups off the chrysene core. For example, while the length of the carbon chains did not confer significant differences, the presence of amine verses amide groups in these chains or modification and/or the presence of the terminal, heterocyclic rings, resulted in striking differences. However, the basis for the cytotoxicity induced by some of the highly active derivatives was not identified.

Therefore, using several of these model compounds, we have now examined the influence of SAR on the induction of the apoptotic pathway in human leukemia Jurkat T cells and have found it to play a crucial role. Of interest, even the most active of these compounds failed to produce any damage in normal, non-transformed natural killer cells.

Materials and methods

Reagents. Fetal bovine serum was purchased from Tissue Culture Biologicals (Tulare, CA). Mixture of penicillin-streptomycin-L-glutamine, MEM sodium pyruvate, MEM

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Key words: polycyclic aromatic hydrocarbons, apoptosis, structure-activity relationships

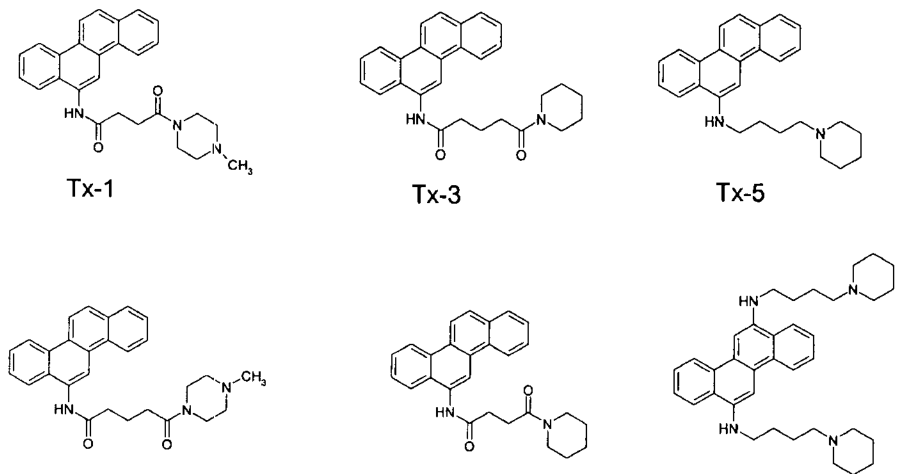

Figure 1. Polycyclic aromatic compounds with varying functional groups. Compounds containing a chrysene moiety with attached amide or amine chains of varying lengths and a terminal heterocyclic group with (Tx-1, Tx-23) or without (Tx-3, Tx-5, Tx-25, Tx-84) an additional amine are shown.

non-essential amino acids, and RPMI were purchased from Invitrogen (Carlsbad, CA). Dimethylsulfoxide (DMSO) and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO). Fluorogenic peptide substrate Ac-DEVD-AMC (for caspase-3/-7 activities) was obtained from Calbiochem (San Diego, CA).

Synthesis of polycyclic aromatic compounds. The compounds used in this study were synthesized as described previously (9).

Cell culture, protein extraction, and Western blot assay. Human leukemic Jurkat T cells and non-transformed natural killer YT cells were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The YT cells were also supplemented with 1 mM MEM sodium pyruvate and 0.1 mM MEM non-essential amino acids solution. These cell lines were maintained at 37˚C in a humidified incubator with an atmosphere of 5% CO_2. Monoclonal anti-mouse IgG-horseradish peroxidase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibody to poly(ADP-ribose) polymerase (PARP) was purchased from Biosource (Camarillo, CA). A whole-cell extract was prepared and Western blotting was performed as described previously (12).

Trypan blue assay. The trypan blue dye exclusion assay was used to ascertain cell death in Jurkat T cells treated with various polycyclic aromatic compounds at various time-points. Briefly, 20 μl of cell suspension was mixed with 20 μl of 0.4% trypan blue dye. The cell/dye mixture was loaded onto a hemocytometer. The number of cells that absorbed the dye and those that excluded the dye were counted to determine the percentage of non-viable cell number to total cell number.

Caspase-3 activity assay. To measure cell-free caspase-3 activity, whole cell extracts (30 μg) from untreated or treated cells were incubated with 40 μM of the fluorogenic substrate caspase-3/-7 (Ac-DEVD-AMC) for 2 h at 37˚C in 100 μl of assay buffer (50 mM Tris, pH 8.0). Measurement of the hydrolyzed AMC groups was performed on a Wallac Victor^3 Multilabel plate reader™ (Perkin-Elmer) with an excitation filter of 380 nm and an emission filter of 460 nm. Changes in fluorescence were calculated and compared with vehicle control treatment and plotted with statistical analysis using Microsoft Excel™ software.

TUNEL assay. Terminal deoxynecleotydyl transferase-mediated dUPT-biotin nick end-labeling (TUNEL) assay was performed using the BD PharMingen, APO-Direct™ kit (San Diego, CA). Cells were treated with varying concentrations, harvested at varying time-points, counted, and washed twice with ice-cold PBS. Briefly, approximately $2x10^6$ cells were fixed in 1% paraformaldehyde for 15 min on ice, washed with PBS, and then fixed again in 70% ethanol at -20˚C overnight. The cells were then incubated in DNA labeling solution [containing terminal deoxynucleotidyl transferase (TdT) enzyme, fluorescein-conjugated dUTP, and reaction buffer] for 90 min at 37˚C. After removing the DNA labeling solution by rinsing cells with rinsing buffer, the cells were incubated with propidium iodide/RNase A solution, incubated for 30 min at room temperature in the dark, and then analyzed by flow cytometry within 3 h of staining. Fluorescence intensity was visualized and estimated for the TUNEL assay using a FACScan (Becton-Dickinson Immunocytometry, CA).

Results

Relationship of structure to cytotoxicity. The compounds utilized in this study possess a chrysene moiety as their core PAH with attached amide or amine chains of varying lengths and a terminal heterocyclic group, with or without an additional amine group (Fig. 1). Their synthesis and their cytotoxicity against a number of human tumor cell lines, using counts of surviving cell numbers, have been reported previously (9-11). In the current study, cytotoxicity against Jurkat T cells was first measured by trypan blue dye exclusion in both dose- and time-dependent assays. Four hours of Jurkat T cell exposure to a concentration of 8 μM of Tx-5 resulted in approximately 45% cell death, which increased to 100% at 8 h (Fig. 2a). In

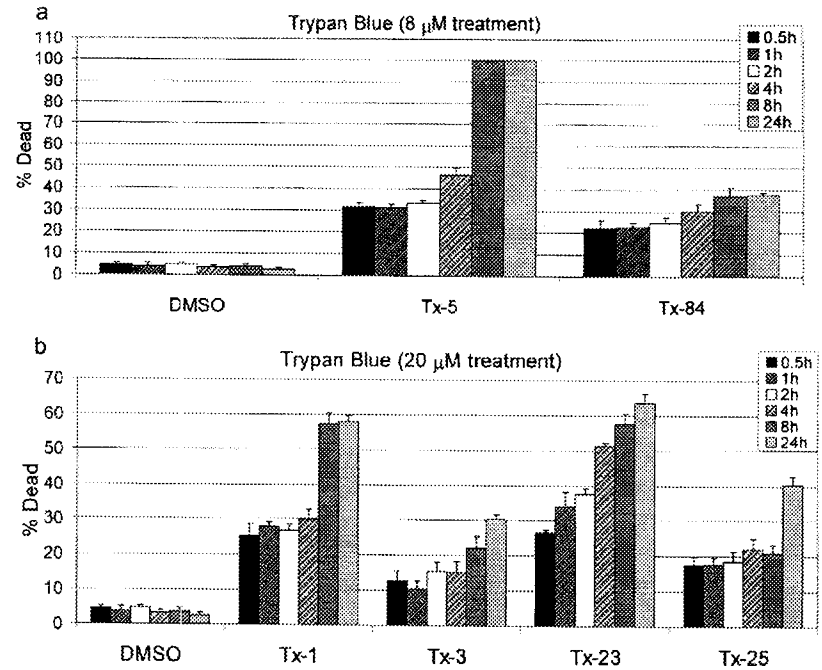

Figure 2. Structure-activity relationships via trypan blue analysis. (a) Jurkat T cells were treated with the solvent (DMSO), 8 μM of Tx-5 or Tx-84, for up to 24 h, followed by trypan blue incorporation assay. (b) Jurkat T cells were treated with the solvent (DMSO) or 20 μM of indicated compound for up to 24 h, followed by trypan blue incorporation assay. The data are represented as the mean number of dead cells over total cell population ± SD and are representative of three independent experiments.

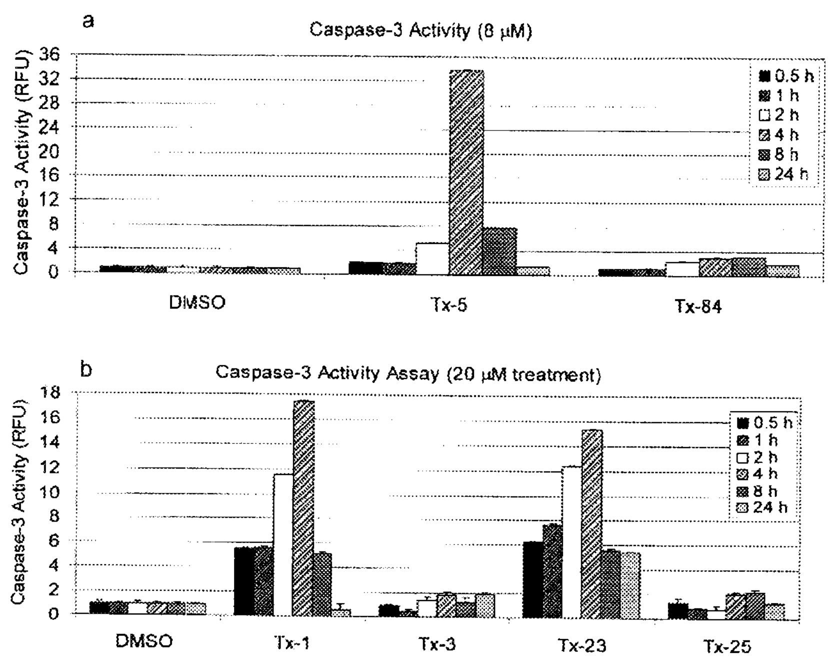

Figure 3. Tx-5 potently induces apoptosis in Jurkat T cells. Jurkat T cells were treated with the solvent (DMSO), (a) 8 μM of Tx-5 or Tx-84 or (b) 20 μM of Tx-1, Tx-3, Tx-23, or Tx-25 for the indicated times, followed by cell-free caspase-3 activity assay. Caspase-3 activity was determined by incubating whole cell extracts with 40 μM caspase-3 substrate and measuring production of hydrolyzed 7-amido-4-methyl-coumarin (AMC) groups using a multi-label plate reader. The results are representative of 3 separate experiments.

contrast, exposure for 4 h to 8 μM Tx-84, which possesses two identical amino chains, resulted in approximately 30% cytotoxicity, which increased by only 10% at 8 h with no further increase through 24 h (Fig. 2a).

Tx-1, Tx-3, Tx-23 and Tx-25 were tested similarly (Fig. 2b). None of these compounds produced significant cytotoxicity of Tx-5 at 8 μM and, therefore, they were tested at 20 μM. At this concentration, Tx-3 and Tx-25 achieved a maximum cytotoxicity of 30% and 40%, respectively. Tx-1 achieved a maximum cytotoxicity of 58% at 8 h and 20 μM while, at this concentration, Tx-23 resulted in a slightly greater cytotoxicity of approximately 63% at 24 h.

Examination of the apoptotic pathway. To determine whether an apoptotic pathway played a role in the cytotoxicity induced in Jurkat T cells by these compounds, activation of caspase-3

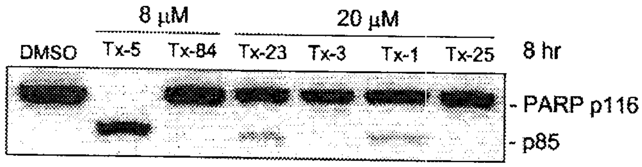

Figure 4. PARP cleavage as a result of apoptotic cell death. Jurkat T cells were treated with the solvent (DMSO), 8 μM of Tx-5 or Tx-84 or 20 μM of Tx-1, Tx-3, Tx-23, or Tx-25 for 8 h. Western blot analysis was performed using specific antibodies to PARP.

was measured during exposure to the 6 compounds. When compared to solvent (DMSO) control values, exposure to Tx-5 resulted in an increase of caspase-3 activity by >30-fold at 4 h (Fig. 3a). Exposure to Tx-84 resulted in a barely detectable increase of 3-fold. To confirm the effect of this degree of caspase-3 activation in Tx-5 treated cells, Western blot analysis was performed to determine the presence of cleaved PARP. Reflecting the cellular impact of this degree of caspase activation resulting from exposure to Tx-5, a significant degree of PARP cleavage was detected in these cells (Fig. 4). In contrast, cells treated with Tx-84 demonstrated no cleavage of PARP, confirming the impression that the level of caspase increase was not significant in terms of resultant cell alteration and damage. These findings also support the impression that the caspase activity measured as a result of exposure to Tx-3 and Tx-25 of at most 2-fold, even at 20 μM treatment (Fig. 3b) was not a significant contributor to cell toxicity. However, at 20 μM, Tx-1 and Tx-23 resulted in a maximal caspase activation of 15- and 17-fold respectively at 4 h and PARP cleavage was observed after 8 h (Fig. 4). These findings further support the possibility that the apoptotic pathway plays a role in the cytotoxicity induced by this series of compounds.

Effects on non-transformed, human natural killer cells. To determine the effect of Tx-5 on normal, non-transformed, natural killer cells, and to extend the examination of its induction of apoptosis, its effects were examined in Jurkat and YT cells using the TUNEL assay. Since ß-lactams such as L-1 have been shown previously to induce apoptosis in human cancer cell lines, but not in normal cell lines (13-15), L-1 was used as a control in this experiment. Jurkat T and YT cells were exposed to 8 μM of Tx-5 or 10 μM L-1 for up to 24 h, harvested, fixed in 70% ethanol, stained (see Materials and methods) and analyzed for TUNEL positivity by flow

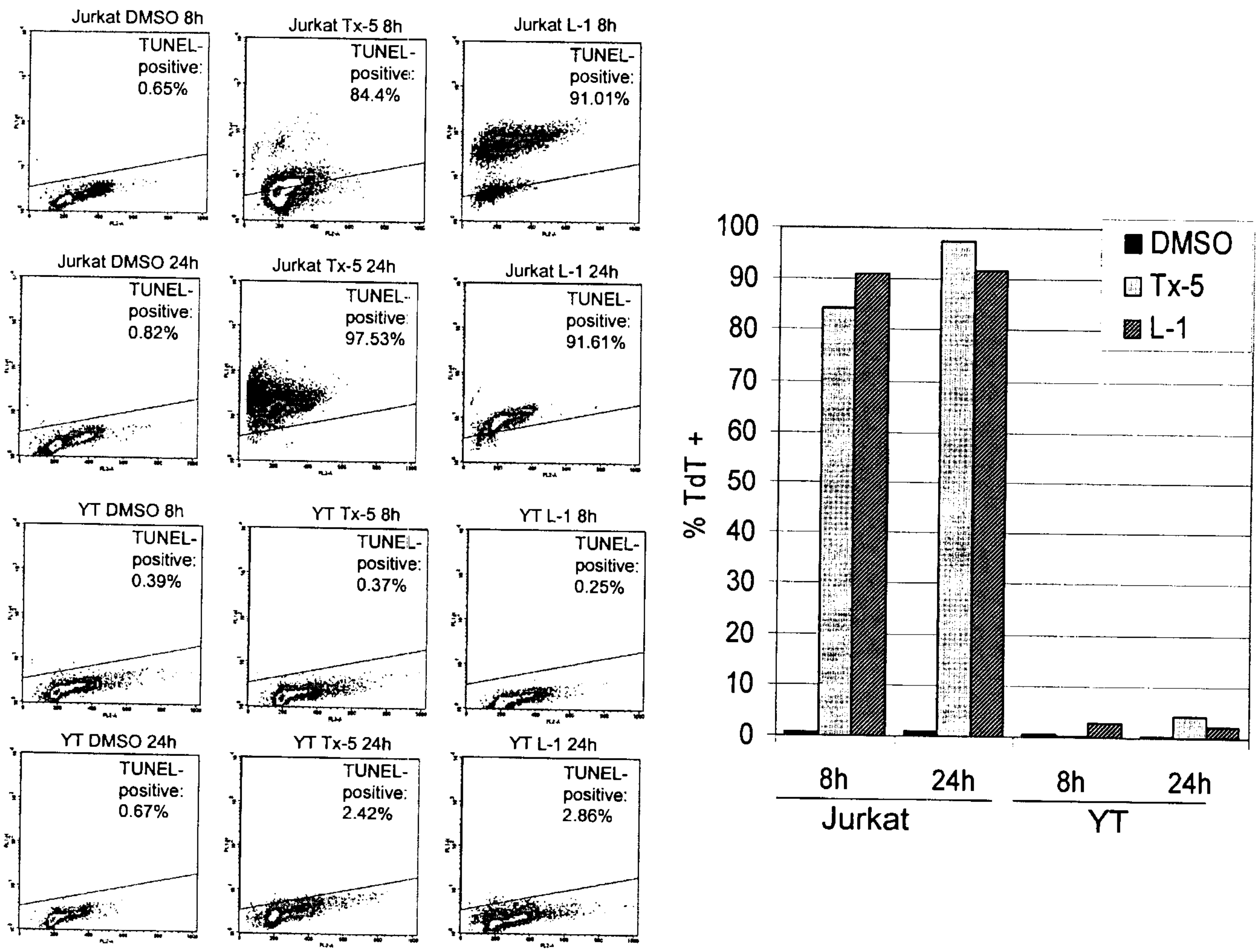

Figure 5. Tx-5 explicitly induces apoptosis in tumor cells. The TUNEL assay was performed by using an APO-Direct kit as described in Materials and methods. Jurkat T and YT cells were treated with the solvent (DMSO), 8 μM Tx-5, or 10 μM lactam-1 for up to 24 h. Fluorescence intensity was estimated for the TUNEL assay using a flow cytometer.

cytometry (Fig. 5). Jurkat cells incubated with Tx-5 were 84.4% TUNEL-positive after 8 h and increased to 98% after 24 h. However, Tx-5 produced no significant difference in TUNEL positivity in YT cells when compared with control cells. L-1 produced similar results.

Discussion

The results of the study of these compounds using trypan blue dye uptake as a measure of cytotoxicity indicate a strong correlation with their chemical structure and, in general, agree with previous results that used counts of surviving cells as a measure of cell death. The most dominant determinant of cell damage was the presence of the diamine chain in Tx-5 as is especially evident when compared with the corresponding diamide derivative, Tx-25. The latter failed to induce any significant degree of caspase-3 activation and significantly lesser level of cell death, even at a 2.5-fold greater concentration. Again, as previously reported, the presence of the terminal N-methpyridinal heterocyclic ring (Tx-1 and Tx-23) was more potent than the terminal ring lacking the methyl group (Tx-3 and Tx-25). In no case did a one C change in chain length alter the effectiveness of the compounds.

One anomalous finding was the significantly lesser cytotoxicity of Tx-84 when compared with Tx-5, a difference that was not previously demonstrated using other human tumor lines, where Tx-84 was equally potent. We must conclude from this finding with Jurkat as the target cell, that the second diamine chain inhibits the expected cytotoxicity by an unknown mechanism. Either this results from some stereo hindrance with the reactive site within the Jukat cell, or suggests that the 12th position of the chrysene ring, which is known to be highly reactive, is required for the cytotoxicity induced by Tx-5. It is, however, further evidence of the high degree of specificity demonstrated by many anti-tumor agents.

The mechanism by which these compounds induced cytotoxicity was not previously known. The high degree of caspase-3 activation, PARP-cleavage, and DNA damage demonstrated by the TUNEL assay as a result of exposure to Tx-5, clearly illustrate the key role played by the apoptotic pathway in the resulting cell death. In addition, the degree of caspase activation induced by Tx-1 and Tx-23, albeit at a higher concentration, suggests that, here too, apoptotic cell death was determinative. These results correlate perfectly with the relative percentages of cell damage as demonstrated in this and previous studies and their correlation with structure-activity.

One of the most striking findings was the absence of effect of Tx-5 against a non-transformed, natural killer cell line, YT. This further supports the probability that the effect of Tx-5 against leukemic Jurkat T cells specifically targets some aspect of tumor cell physiology, which strengthens its possible role in clinical treatment.

Finally, while there was no significantly detectable increase in caspase-3 activation at 8 μM Tx-84 or at 20 μM Tx-3 and Tx-25, each of these compounds induced approximately 30-40% cell death. It should not come as a surprise that this finding suggests that alternate pathways exist for inducing cell damage, such as direct interaction with the cells' surfaces, as has been previously recognized.

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RELATIONSHIPS OF STRUCTURES OF N-METHYLTHIOLATED BETA-LACTAM ANTIBIOTICS TO THEIR APOPTOSIS-INDUCING ACTIVITY IN HUMAN BREAST CANCER CELLS

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Activation of the cellular apoptotic program is a current strategy for the prevention and treatment of human cancer including breast cancer. The beta-lactam antibiotics have played an essential role in treating bacterial infections without causing toxic side effects for the past 60 years. We hypothesize that active N-thiolated beta-lactams can target a tumor-specific protein(s) and selectively induce apoptosis in human breast cancer but not normal cells.

To test this hypothesis, we designed and synthesized numerous new N-thiolated beta-lactams analogs, evaluated potencies of these synthetic beta-lactams to inhibit proliferation and induce apoptosis in human breast cancer cells, and investigated whether these beta-lactams can induce apoptosis selectively in breast tumor vs. normal cells and the involved molecular mechanisms.

We tested a library of lactam compounds and found that many of them are able to inhibit proliferation and induce apoptosis in human breast cancer cells in a time- and concentration-dependent manner. These active beta-lactams are capable of inducing DNA strand breakage, inhibiting DNA replication, and inducing p38 MAP kinase activation. Consistent with the idea that these beta-lactam antibiotics are potent anti-cancer agents, the active analogs are also capable of inhibiting colony formation potential of breast cancer cells. Furthermore, the active beta-lactams have much reduced effects on human normal or non-transformed cells. Our findings also yielded several important structure-activity relationships (SARs). The N-methylthio group is necessary for the apoptosis-inducing activity. Also observed is the inverse relationship between the number of carbon atoms off the N-thio group and apoptotic activity. Substitutions to C3 reveal that as the substituents increase in size or in polarity, the efficacy of the compound decreases. Therefore, the overall size of the beta-lactam is important, possibly indicating steric hindrance with the cellular target or permeability to the cell membrane. We also found that the stereochemistry of the beta-lactams play in important role in their potency. The 3R,4S enantiomers are more efficacious than the 3S,4R isomers, which may indicate a more favorable configuration for target interaction.

Our future studies will focus on the biochemical target of N-thiolated beta-lactams, whether the N-thiolated beta-lactams can induce apoptosis selectively in breast tumor vs. normal breast cells, and whether the in vivo apoptosis-inducing ability of the N-thiolated beta-lactams is related to their cancer-preventive and anti-tumor activities using nude mice bearing human breast tumors. These studies should provide strong support for proof-of-concept of the potential use of these N-thiolated beta-lactams in breast cancer prevention and treatment.

The U.S. Army Medical Research and Materiel Command under DAMD17-03-1-0175 and W81XWH-04-0688 supported this work.

From: AACR [mailto:AACR@DBPUB.COM]
Sent: Tue 1/17/2006 1:00 AM
To: Landis, Kristin
Subject: 2006 AACR Annual Meeting - Abstract Number #2093

January 2006

Re: 2006 AACR Annual Meeting in Washington, DC

Temporary Abstract Number #2093
Title: Computational modeling and biological evaluation of methylated (-)-EGCG analogs

Dear Dr. Landis-Piwowar:

Your above-referenced abstract has been scheduled for oral presentation in a Minisymposium session at the 2006 AACR Annual Meeting in Washington, DC and will be published in the 2006 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session ID: Prevention Research 10
Session Date and Start Time: Tuesday, April 4, 2006 2:00 PM
Permanent Abstract Number: 4897

Please refer to the printed Final Program (distributed onsite) or the online Annual Meeting Itinerary Planner [available in early March through the AACR Website at http://www.aacr.org] for the exact location of your presentation.

Instructions for Presenters in Minisymposia can be found on the 2006 AACR Annual Meeting home page: http://www.aacr.org/page5395.aspx

Minisymposium presenters at the AACR Annual Meeting must register for the full meeting at the rate appropriate to their membership status and obtain their own hotel accommodations. Registration and housing information are included below:

Advance Registration Deadline: February 24, 2006
Online Registration System:
http://www.aacr.org/page5316.aspx

Housing Deadline: March 8, 2006
Online Housing System:
http://www.aacr.org/page5289.aspx

Online Airline Reservation System:
http://www.aacr.org/page5141.aspx

For more information, visit the 2006 AACR Annual Meeting home page at:
http://www.aacr.org/page5295.aspx.

Thank you for your participation in the 2006 AACR Annual Meeting.

Sincerely,

Daniel A. Haber, M.D., Ph.D.
Program Committee Chairperson

PLEASE NOTE: This document is your official notice of acceptance. No separate letter of acceptance will be mailed.

Computational Modeling and Biological Evaluation of Methylated (-)-EGCG Analogs

Kristin R. Landis-Piwowar, Sheng Biao Wan, Kenyon G. Daniel, Di Chen, Tak Hang Chan, and Q. Ping Dou

The anti-cancer and cancer-preventive effects of green tea and its main constituent (-)-epigallocatechin-3-gallate [(-)-EGCG] are well documented by a variety of studies, including epidemiological, cell culture, animal, and clinical. While (-)-EGCG remains the most potent polyphenol in green tea, it is particularly unstable under neutral or alkaline conditions (*i.e.* physiologic pH) and may be modified by methylation, glucuronidation and sulfonation. In order to understand the significance of methylation on cancer-preventative effects of tea polyphenols, we synthesized several (-)-EGCG and (-)-ECG analogs with various number of methyl groups (mono-, di-, and tri-) attached to the B- and/or D-rings. Additionally, we synthesized putative prodrugs that contain protective peracetate groups (removable by cellular cytosolic esterases) in place of the remaining hydroxyl groups. The structure-activity relationships (SARs) were studied by both *in silico* computational modeling of the methylated EGCG analogs to the proteasome active site and their *in vitro* inhibitory potencies against a purified 20S proteasome. While the number of methyl groups was increased, a gradual decrease was observed in the probability of methylated (-)-EGCG analogs to bind *in silico* to the active site of the proteasome. Consistently, as the number of methyl groups increased on the (-)-EGCG molecule, the *in vitro* proteasome-inhibitory potencies were also decreased. When human leukemic Jurkat T cells were tested, the pro-drug of the mono-methylated (-)-EGCG analog caused greater proteasome inhibition and apoptosis than the pro-drug of the tri-methylated (-)-EGCG analog. Our data suggest that methylation of (-)-EGCG lessens its proteasome-inhibitory ability that might lead to decreased cancer preventative effects.

From: Lanette Rowland [mailto:lrowland@med.wayne.edu]
Sent: Mon 5/23/2005 12:17 PM
To: Hurst, Newton; Krueger, Sarah; Landis, Kristin; Locke, Deborah
Cc: Brooks, Sam
Subject: Training Grant - Payroll Transition

Dear New Training Grant Students:

Congratulations on being awarded the "Ruth L. Kirschstein National Research Service Award (NRSA)" by the National Cancer Institute. This is an honor and should be included in your CV.

Your training grant assignments starts on September 1, 2005 and will run for two years. Paperwork will be prepared in July and I will ask you to answer some questions by e-mail and then come to 329 Lande to sign the paperwork. You will no longer be on WSU payroll and taxes will not be taken.

So you are able to plan your financial expenses for the summer and save for the two paydays that will include less money, please see below. Your assignment of the GRA (Graduate Research Assistant) ends on August 18, 2005 (Deborah, please check your letter of offer from last year for your end date).

Sept 7 payday - you will receive only 4 days of pay (Aug. 15-18)
Sept 21 payday - you will only receive 2 days of pay at new training grant rate (Sept 1 and 2)
Oct 5 payday - you will receive 10 days of regular pay.

At the end of two years of assignment, you will catch-up as you come off the training grant on August 31 and get assigned as a GRA effective Aug. 19 or so.

If you have any questions, please feel free to contact me. Thanks.
****

Lanette Rowland
Administrative Assistant
WSU - Graduate Program in Cancer Biology
329 Lande Building
Detroit, MI 48202
313-577-1065
FAX 313-577-4112
ad3340@wayne.edu

****

gsrdash

WAYNE STATE UNIVERSITY GRADUATE STUDENT RESEARCH DAY 2005

Certificate of Recognition Presented to

Kristin Jandis-Piwowar

First Place

Oral Presentation Session 3

WAYNE STATE UNIVERSITY

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Kenneth C. Palmer, PhD

Assistant Dean for Graduate Programs

The School of Medicine

Presents this Certificate to

Kristin R. Landis-Piwowar

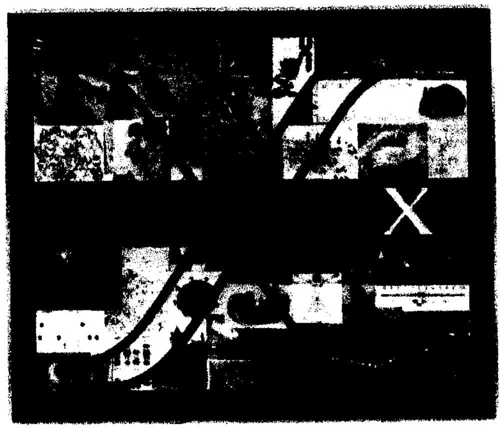
In Recognition of your Exceptional

Academic and Research Accomplishments

WAYNE STATE UNIVERSITY
SCHOOL OF MEDICINE

Robert R. Frank
Dean of the School of Medicine

12/1/05
Date



1ST PRIZE FOR ORAL PRESENTATION
AT WAYNE STATE UNIVERSITY 10TH ANNUAL GRADUATE STUDENT RESEARCH DAY, SEPTEMBER 21, 2006

Presented to:

Kristin Landis-Piwowar

WAYNE STATE UNIVERSITY
SCHOOL OF MEDICINE
1868

Kenneth C. Palmer, PhD
Assistant Dean for Graduate Programs

CURRICULUM VITAE

Q. Ping Dou, Ph.D.

Date of Preparation: March 18, 2007

Signature:__________________

OFFICE ADDRESS

The Prevention Program
Barbara Ann Karmanos Cancer Institute, and
Department of Pathology
Wayne State University School of Medicine
640.1 Hudson-Webber Cancer Research Center
4100 John R Road
Detroit, MI 48201-2013
Telephone: 313-576-8301 (Office)
313-576-8264/-8025/-8026/-8247/-8249/-8250/-9397 (Lab)
313-576-8299 (Adm. Assistant)
Fax: 313-576-8307 (Office)
313-576-8306 (Adm. Assistant)
E-mail: doup@karmanos.org

EDUCATION:

B.S. in Chemistry, Shandong University, Jinan, Shandong, People's Republic of China, 1981
Ph.D. in Chemistry, Rutgers University, Piscataway, NJ (Mentor: Kuang Yu Chen), 1988

TRAINING:

Postdoctoral Research Fellow in Molecular Biology and Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA (Mentor: Arthur B. Pardee), 1988-1992

FACULTY APPOINTMENTS:

Instructor, Department of Medicine, Harvard Medical School, at Dana-Farber Cancer Institute and Beth Israel Hospital, Boston, MA, 1992-1993

Assistant Professor, Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 1993-1998

Assistant Professor, Biochemistry and Molecular Genetics Graduate Training Program, Interdisciplinary Biomedical Graduate Program, University of Pittsburgh School of Medicine, Pittsburgh, PA, 1997-1998

Member, Experimental Therapeutic Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA, 1993-1998

Member in Residence, Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida, 1998-2003

Associate Professor, Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine, Tampa, Florida, 1998-2003

Associate Professor, Department of Interdisciplinary Oncology, University of South Florida College of Medicine, Tampa, Florida, 2000-2003

Member, the Institute for Biomolecular Science, University of South Florida, Tampa, Florida, 1998-2003

Assistant Program Leader and Scientific Member, Population Studies and Prevention Program, Barbara Ann Karmanos Cancer Institute, Detroit, MI, 2003-present

Leader and Scientific Member, Prevention Program, Barbara Ann Karmanos Cancer Institute, Detroit, MI, 2003-present

Full Professor (with Tenure), Department of Pathology, Wayne State University School of Medicine, Detroit, MI, 2003-present

Full Professor, Cancer Biology Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2003-present

Full Member, Gene Regulation and Genetics Research Program, Institute of Environmental Health Sciences, Wayne State University, Detroit, MI, 2004-present

Member, Scientific Leadership Council (SLC), Barbara Ann Karmanos Cancer Institute, Detroit, MI, 2004-present

Member, the NanoSciences Institute, Wayne State University, Detroit, MI, 2005-present

Honorary Professor in the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China, 2006-

Honorary Professor in the Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, 2006-

Honorary Professor, Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, Hubei, China, 2006-

MAJOR PROFESSIONAL SOCIETIES:

American Association for Cancer Research, Inc.
American Association for the Advancement of Science
American Society for Biochemistry and Molecular Biology
American Society for Pharmacology and Experimental Therapeutics
Society of Chinese Bioscientists in America
International Society for Study of Comparative Oncology, Inc.
American Chemistry Society
New York Academy of Sciences (1995-1998)

HONORS AND AWARDS:

Summer Research Prize in recognition of outstanding accomplishments in research. Rutgers University, 1988

Biochemical Research Support Grant Award. Dana-Farber Cancer Institute, 1991

Barr Program Small Grant Award. Dana-Farber Cancer Institute, 1992

Co-Discussion Leader, University of Pittsburgh Cancer Institute Scientific Retreat, 1995

NIH Director James A. Shannon Award, 1 R55 AG/OD13300-01, 1995-1997

NIH FIRST Award, R29 AG13300-05, 1996-2001

A Predoctoral Trainingship in Breast Cancer Biology and Therapy from the United States Army Medical Research, Development, Acquisitions, and Logistics Command (to Cheryl L. Fattman), 1997-1999

The Best Poster Presentation (An B *et al.*), Scientific Retreat, Department of Pharmacology, University of Pittsburgh School of Medicine, 1997

Chairman for Session of Clinical Oncology/Apoptosis. 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

Award for the Best Abstract, 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

Cheryl L. Fattman, Ph.D. Graduation with Honor from University of Pittsburgh (mentor: Q. Ping Dou), 1999

Moffitt's Cancer Center Director's Award (for the article published by Li B and Dou QP in Proc. Natl. Acad. Sci. USA, 2000; 97: 3850-3855). Moffitt Cancer Center & Research Institute, 2000

An AACR-AFLAF Scholar-in Training Award ($400 to Aslam Kazi/ mentor: QP Dou), for a selected poster (Abstract #788, Kazi A , Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X_L Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

Abstract was chosen as one of the selected few for News Briefing (Abstract #788, Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X_L Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

Nominee of Team Award (Cancer Control Program), 2002

Kenyon G. Daniel, Ph.D. a Winner of the 2004 Outstanding Dissertation Award from University of South Florida (Major Professor: Q. Ping Dou), 2004

Invited Visiting Professor in the Department of Urology at the University of California San Francisco and San Francisco VA Medical Center (April 28, 2005), with seminar presentation, "Searching for novel polyphenol proteasome inhibitors for cancer prevention and treatment"

Training Grant (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University, 2005-2007

First Place, Oral Presentation (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University School of Medicine, Graduate Student Research Day, September 22, 2005. "Molecular Studies for the Regulation of Green Tea Polyphenol Biological Function by the Polymorphic Gene Product Catechol-O-Methyltransferase".

Awardee (Kristin Landis; Mentor: Q. Ping Dou) for the Honors Recognition Program for Wayne State University School of Medicine, Wayne State University, 2005

Invited Visiting Professor in the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China (April 19-20, 2006), with seminar presentation, "Cancer Prevention and the Role of Environmental Factors". Received Honorary Professor Title.

Invited Visiting Professor in the Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China (April 24-25, 2006), with seminar presentations, "Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer" and "Discovery of Novel Small Molecules from Nature and Laboratories for Cancer Therapies"

The First Place Poster Award (Huanjie Yang , Di Chen, Qiuzhi Cindy Cui, Xiao Yuan, and Q. Ping Dou). The 6th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research , University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006. "Celastrol, A Triterpene Extracted From The Chinese Thunder Of God Vine, Is A Potent Proteasome Inhibitor And Suppresses Human Prostate Cancer Growth In Nude Mice".

Honorary Professorship,* Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, Hubei, China *(October, 2006), with seminar presentation, "Discovery of Novel Small Molecules for Cancer Therapies".

Nominee of the 2007 AACR Landon Prize for Basic Research.

First Place, Oral Presentation (Kristin Landis; Mentor: Q. Ping Dou). Wayne State University School of Medicine, Graduate Student Research Day, September 21, 2006. "A Novel Pro-drug of Green Tea Catechin (-)-EGCG as a Potential Anti-Breast Cancer Agent".

Co-Session Chairman. The 3rd International Symposium on Persistent Toxic Substances, Beijing, China, October 22-25, 2006

Winner of Karmanos Cancer Center Director's Award (for the article published by Yang HJ, Chen D, Cui QC, Yuan X, and Dou QP in* Cancer Research *66, 4758-4765, 2006). Karmanos Cancer Institute, 2006

Awardee (Kristin Landis; Mentor: Q. Ping Dou) for the Honors Recognition Program for Wayne State University School of Medicine, Wayne State University, 2006

Invited Speaker. Disulfiram. Molecular Therapeutics of Cancer, Gordon Research Conference, Colby-Sawyer College, New London, New Hampshire, July 22-27, 2007.

Invited Speaker. Green Tea Polyphenols as a Natural Tumor Cell Proteasome Inhibitor. The 3rd International Symposium on Functional Foods, PLANT POLYPHENOS, "*What would life be without plant polyphenols*?" Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, P.R. China, April 03-05 2008

Invited member of the International Advisory Committee for the International Conference on Nutrition and Cancer, Elazig, Turkey, May 20-23, 2008

SERVICE:

Professional Service Activity

Tours for University of Pittsburgh Cancer Institute

Tours for Drug Discovery Program Moffitt Cancer Center & Research Institute

Advisor for Project LINK (Leaders In New Knowledge) Students

Advisor for Moffitt Summer Interns

Advisor for Undergraduate Student Honor's Thesis Research

Member, the Scientific Advisory Board, Torrey Pharmaceuticals, LLC, San Diego, CA, 2000-present

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 3, 2004, April 2, 2005

Presentation to Cancer Biology Program Candidates, Karmanos Cancer Institute and Wayne State University, March 25, 2006 (Kristin Landis and Vesna Minic from Dr. Dou's laboratory)

Preferred Consultant, MEDACorp, Boston, Massachusetts, 2004-present

Speaker (Kristin Landis from the laboratory of Q. Ping Dou), "Friends Raising Funds" program, March 5th, 2006, the Powerhouse Gym, West Bloomfield, MI.

Invited Speaker, "Green Tea and Cancer Prevention", "The Day of Wellness" Program, September 16, 2006, the Grosse Pointe War Memorial, Grosse Pointe, MI.

Moderator, Population Studies and Prevention Program Scientific Retreat, Lung Cancer session, Karmanos Cancer Institute and Wayne State University, September 15, 2006

Invited Speaker, Green Tea and its Role in Cancer Prevention. Dow and Towsley Foundation Visit, Karmanos Cancer Institute Research, Detroit, MI, October 11, 2006

Invited member of the International Advisory Committee for the International Conference on Nutrition and Cancer, Elazig, Turkey, May 20-23, 2008

Journal/Editorial Activity

Editorial Board Memberships

Invited member of the Editorial Board of the *Oncology Reports,* 1996-present

Invited member of the Editorial Board of *Frontiers In Bioscience,* 1997-present

Invited member of the Editorial Board of *LifeXY* (Currently *International Archives of Biosciences*), 2001-present

Invited panel evaluator of *Current Drugs,* 2001-present

Invited member of *The Science Advisory Board,* 2002-present

Invited managing editor of *Frontiers In Bioscience,* 2003-present

Invited managing editor of *Frontiers In Bioscience* for a special issue of “Potential Molecular Targets for Chemoprevention”, 2004-present

Reviewer for Journal Manuscripts

Proceeding of National Academy of Sciences USA
FASEB J
Oncogene
Chemistry & Biology
Cancer Research
Clinical Cancer Research
Molecular Cancer Therapeutics
Cell Death & differentiation
Molecular Pharmacology
Journal of Pharmacology & Experimental Therapeutics
Exp Cell Res.
J Cellular Physiology
Drug Discovery Today
Microbes and Infection
Leukemia
Cancer Letters
FEBS Letters
Carcinogenesis
International J. Oncology
Breast Cancer Research and Treatment
J. Cell. Biochemistry
Biochemical Pharmacology
BMC Cancer
Life Sciences
Cancer Chemotherapy and Pharmacology
Lipids
European Journal of Medicinal Chemistry
European Journal of Cancer
Endocrine
Apoptosis
Arch Biochem Biophys
Journal of Pharmacy and Pharmacology
Head & Neck
Journal of Agriculture and Food Chemistry
Obesity Research
Molecular Nutrition and Food Research
Natural Immunity
Molecular Biology Reports
Gene Therapy
Cellular and Molecular Life Sciences
Expert Opinion on Investigational Drugs
Expert Opinion on Drug Metabolism & Toxicology

Expert Review of Anticancer Therapy
Expert Review of Proteomics
Evidence-Based Integrative Medicine
Life XY (Currently *International Archives of Biosciences*)
The Pittsburgh Undergraduate Review

Reviewer for Grant Applications

Competitive Medical Research Fund (Ad Hoc Reviewer), University of Pittsburgh School of Medicine, 1996

Competitive Medical Research Fund, University of Pittsburgh School of Medicine, 1997

Central Research Development Fund, University of Pittsburgh, 1997

National Science Foundation, 1998

Merit Review Subcommittee for Oncology, the Veterans Affairs Medical Research Programs, US Department of Veterans Affairs, 1999-2005

Member, Cancer Study Section, The Tobacco-Related Disease Research Program, University of California, San Francisco, CA, 2004-present

Invited Reviewer, Biotechnology and Biological Sciences Research Council Research Grant, United Kingdom, 2004, 2006

Invited Proposal Reviewer, The Kentucky Science and Engineering Foundation's R&D Excellence Program, Lexington, KY, 2004, 2005

Invited Reviewer, the Strategic Research Initiative Awards, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2005

Invited Reviewer, the Seed Money Grants, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2006

Invited Reviewer, the European Commission's Research Program on "Combating Cancer" - Apoptosis, Brussels, Belgium, 2006

Other Professional Related Service

Faculty reviewer for applications to University of Pittsburgh and University of Pittsburgh Cancer Institute

Faculty reviewer for applications to Moffitt Cancer Center & Research Institute and University of South Florida

Faculty reviewer for applications to Karmanos Cancer Institute and Wayne State University (2003-)

Faculty reviewer for Deputy Director/Associate Center Director Candidates to Karmanos Cancer Institute and Wayne State University (2004)

Faculty reviewer for Lambert – Webber Endowed Chair, Division Chief, Hematology and Oncology, Department of Internal Medicine, Wayne State University, Program Leader, Developmental Therapeutics, Karmanos Cancer Institute (2005)

National and International Boards and Committees

Ad Hoc Reviewer, National Science Foundation, 1998

Ad Hoc Reviewer, Merit Review Subcommittee for Oncology, the Veterans Affairs Medical Research Programs, US Department of Veterans Affairs, 1999-2001

Member, Department of Veterans Affairs (VA) Medical Research Service Merit Review Subcommittee for Oncology, 2001-2005

Member, the Scientific Advisory Board, Torrey Pharmaceuticals, LLC, San Diego, CA, 2000-present

Council Member, Gerson Lehrman Group's Council of Healthcare Advisors, New York, NY, 2004-present

Member, Cancer Study Section, The Tobacco-Related Disease Research Program, University of California, San Francisco, CA, 2004-present

Invited Reviewer, Biotechnology and Biological Sciences Research Council Research Grant, United Kingdom, 2004, 2006

Preferred Consultant, MEDACorp, Boston, Massachusetts, 2004-present

Judge, Cell Biology & Cell Signaling Section, 2nd Annual Research Symposium, Henry Ford Health System Academic Affairs, Detroit, MI, April 15, 2005

Invited Reviewer, the European Commission's Research Program on "Combating Cancer" - Apoptosis, Brussels, Belgium, 2006

Invited member of the International Advisory Committee for the International Conference on Nutrition and Cancer, Elazig, Turkey, May 20-23, 2008

State and Local Boards and Committees

Department of Pharmacology, University of Pittsburgh School of Medicine

Comprehensive Examination Committee, Department of Pharmacology, University of Pittsburgh School of Medicine, 1993-1998

Committee of Graduate Studies, Department of Pharmacology, University of Pittsburgh School of Medicine, 1994-1998

Chairman of Graduate Evaluations, Department of Pharmacology, University of Pittsburgh School of Medicine, 1994-1998

NIH Predoctoral Training Grant Selection Committee, 1995

Director of Departmental Seminar Program, 1997

University of Pittsburgh School of Medicine

Competitive Medical Research Fund Review Committee (Ad Hoc Reviewer), University of Pittsburgh School of Medicine, 1996-1997

The Graduate Progress Evaluation Committee, University of Pittsburgh School of Medicine, 1997

Central Research Development Fund, University of Pittsburgh, 1997

University of South Florida and Moffitt Cancer Center & Research Institute

Member, Search Committee for Assistant Professor position in Molecular, Epidemiology Program of Cancer Control Division at Moffitt Cancer Center & Research Institute, 2001

Member, Rb Club, Moffitt Cancer Center & Research Institute, 2001-2002

Member, The Summer Intern Program Committee at Moffitt Cancer Center & Research Institute, 2002

Member, Preliminary Data Club, Moffitt Cancer Center & Research Institute, 2002-2003

Member, Proteomics Task Force Committee, Moffitt Cancer Center & Research Institute, 2002

Member, Search Committee for Structural Biology Faculty position in Drug Discovery Program at Moffitt Cancer Center & Research Institute, 2003

Member, Search Committee for the Cancer Prevention Faculty position in Molecular Epidemiology Program of Cancer Control Division at Moffitt Cancer Center & Research Institute, 2003

Member, Search Committee for Chemistry Faculty position in Drug Discovery Program at Moffitt Cancer Center & Research Institute, 2003

Member, SPARK (Summer Program for the Advancement of Research Knowledge) Selection Committee at Moffitt Cancer Center & Research Institute, 2003

Wayne State University and Karmanos Cancer Institute

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 3, 2004

Graduate student recruitment faculty for Cancer Biology Program Karmanos Cancer Institute and Wayne State University, April 2, 2005

Invited Reviewer, the Strategic Research Initiative Awards, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2005

Invited Judge, the 2nd Annual Research Symposium, Henry Ford Health System, Detroit, MI, 2005

Invited Reviewer, the Seed Grants, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, 2006

Co-Director and Member of a P01 Group, "AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer", Wayne State University and Henry Ford Health System, Detroit, MI, 2004-present

Speaker (Kristin Landis from the laboratory of Q. Ping Dou), "Friends Raising Funds" program, March 5th, 2006, the Powerhouse Gym, West Bloomfield, MI.

Member, HFHS/WSU Prostate Journal Club, 2003-

Co-Director and Member of a P01 Group, "AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer", Wayne State University and Henry Ford Health System, Detroit, MI, 2004-present

Co-Director and Member of a P01 Group focusing on nutritional agents, "Perturbation of molecular signaling by nutritional agents for cancer prevention and therapy", Prevention Program, Karmanos Cancer Institute, Wayne State University, Detroit, MI, 2007-present

Dr. Dou has the following responsibilities:

As the leader of the Molecular Prevention sub-Program of Karmanos Cancer Institute, Dr. Dou has the following responsibilities:

(1) Assist the Program Leader in the entire Population Studies and Prevention program and provide leadership in the Molecular Prevention sub-program, particularly with respect to basic science research, molecular targeting and chemoprevention;
(2) Participate in Population Studies and Prevention leadership meetings;
(3) Participate in updating and maintaining strategic planning for the Prevention sub-program;
(4) Facilitate inter- and intra-programmatic interactions between the Prevention faculty members and members of Population Studies, Communication & Behavioral Oncology, and other cancer center programs;
(5) Facilitate and direct nutrition/prevention-related joint grants and program projects;
(6) Organize and lead the monthly Nutrition & Cancer Working Group meetings;
(7) Recruit new members into the Molecular Prevention sub-program and mentor junior faculty;
(8) Advocate for shared facilities that meet the needs of the Prevention members;
(9) Develop the Prevention sub-program into an independent program in the next three years.

TEACHING:

Years at Wayne State University: Since August 1, 2003
Years at Other Universities:

Harvard Medical School, 1 year
University of Pittsburgh, 5 years
University of South Florida, 5 years

Courses Taught at Wayne State University

2003- CB 7250: CANCER CONTROL. 3 credits. 20 students

2004 CB 7230: BREAST CANCER. 2 credits. 10-12 students (December 8, 2004, 10:00 AM- 12:00 PM, 1140 Scott Hall).

2005- CB 7700: RECENT DEVELOPMENT IN CANCER BIOLOGY. 2 credits. ~20 students (April 11, 2005; Oct 24, 2005; Feb 12, 2007)

Courses Taught at University of South Florida

1999-2001 BCH 6411: Molecular Biology. Lecture. 3 credits. 25-30 students

2001- Cancer Biology I Course Lecture. 3 credits. ~10 students

Courses Taught at University of Pittsburgh

1993-1998 MS MIC 2355: Advanced Molecular Genetics. Lecture and Paper Discussion. 3 credits. 8-16 students

1993-1998 PHL 3510: Receptors and Signal Transduction. Lecture and Paper discussion. 3 credits. 10-15 students

1993-1998 2563: Cancer Pharmacology. Lecture. 3 credits. ~5 students

1993-1998 Medical Student Program: Problem-Based Learning Sessions. 8-10 students

1995 Medical Student Program: Pharmacology Conference. ~20 students

1997 Medical Student Program: Neoplasia and Neolastic Disease. 16 students

1996-97 The Pennsylvania Governor's School Program. 6-8 students

1997 Foundations of Biomedical Science. Small group conference. 3 credits. ~8 students

Undergraduate and Graduate/Medical Student Supervision

1994 Chen Yu, Harvard University, ASPET undergraduate

1995 Peggy Lin, Penn State-Jefferson

1995 Bill Wang, California University of PA

1995 Vivian Lui, Department of Pharmacology, University of Pittsburgh School of Medicine, one lab rotation

1996 Toni A. Termin, Saint Vincent College, ASPET undergraduate

1996 Kirk E. Dineley, Department of Pharmacology, University of Pittsburgh School of Medicine, two lab rotations

1996-1999 Cheryl Fattman, Department of Pharmacology, University of Pittsburgh School of Medicine. Ph.D., Dissertation Title: "Molecular mechanisms for apoptosis-associated the retinoblastoma protein (RB) internal cleavage". Graduation with Honor (Mentor: Q. Ping Dou). Currently working as a postdoctoral fellow in Department of Pathology, University of Pittsburgh School of Medicine

1997 Lachelle Sussman, University of New York at Buffalo, ASPET Undergraduate, University of Pittsburgh School of Medicine, one lab rotation

1997 Kristin S Morrow, Department of Biology University of South Florida, master graduate student

1998- Yaser S. Bassel, University of South Florida College of Medicine, medical student

1998- Jason A. Evangelista, University of South Florida College of Medicine, medical student

1998- Joseph J. Kavanagh, University of South Florida College of Medicine, medical student

1998- Alexander Paloma, University of South Florida College of Medicine, medical student

1998- Gregory A. Russell, University of South Florida College of Medicine, medical student

1999-2002 David M. Smith, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab (graduated in 2002). Dissertation Title: “Mechanistic Studies on Tumor Cell Cycle Disruption and Apoptosis by Green Tea Polyphenols and N-Thiolated beta-Lactams”. Currently working as a postdoctoral fellow in Dr. Fred Goldberg’s laboratory at Harvard Medical School)

1999 Lisa Smith, Department of Biology University of South Florida, undergraduate student (currently a graduate student in University of North Carolina)

1999 Jessica Hu, Harvard University, undergraduate student

1999 Daniel Lorch, University of Florida, undergraduate student

1999 Sun Hee Rim, Hillsborough High School, student

1999 Alvin Jones, Land O’Lakes High School, student

1999 Kristie Main, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation

1999-2000 Kenyon Daniel, Department of Biology University of South Florida, undergraduate student. Research for Honor’s Thesis

2000 Lisa Smith, Moffitt Summer Intern, Department of Biology University of South Florida, undergraduate student (current graduate student at University of North Carolina)

2000-2002 Marie Bosley, Project LINK (Leaders In New Knowledge) Student and a Moffitt Summer Intern, Department of Biology University of South Florida, undergraduate student

2000 John (Chilu) Chen, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation

2001 Jonathan S. Anderson, Moffitt Summer Intern, Zoology, University of Florida, undergraduate student

2001 Kyleen Charlton, Moffitt Summer Intern, Boston College, undergraduate student

2001-2002 Priyanka Kamath, Volunteer, Hillsborough High School, high school student

2000-2004 Kenyon Daniel, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab (graduated in 2004). Dissertation Title: “Strategies for Cancer Therapy Through Regulation of Apoptotic Proteases”. Currently working as a postdoctoral fellow in my laboratory at Karmanos Cancer Institute, Wayne State University

2001-2004 Deborah Kuhn, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab (graduated in November of 2004). Dissertation Title: “Novel Approaches to Targeting Tumor Cell Apoptotic Signaling Pathways”. Currently working as a postdoctoral fellow in University of North Carolina.

2002 Naveen Kumar, Moffitt Summer Intern, New York University, undergraduate student

2002 Randy Hill, Moffitt Summer Intern, University of Wisconsin, undergraduate student (currently a graduate student in University of Wisconsin)

2002 Seth Pross, Moffitt Summer Intern, Hillsborough High School, high school student (currently a graduate student in University of Pennsylvania)

2002-2003 Audrey Burns, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab

2002-2003 Sam Falsetti, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation and Ph.D. student in my lab

2002 Jennelle McQuown, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Medicine, one lab rotation

2002-2003 Daniel Urbizu, Project LINK (Leaders In New Knowledge) Student, Department of Biology University of South Florida, undergraduate student
2003 Thomas Lendrihas, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, one lab rotation
2003 Seth Pross, Moffitt Summer Intern, University of Pennsylvania, undergraduate student
2003 Shuojing Song, Moffitt Summer Intern, C. Leon King High School, high school student (currently an undergraduate of MIT)
2003 R. Hope Harbach, Summer Student, Department of Chemistry, Eckerd College, undergraduate student
2003 Daniel Urbizu, Summer/Project LINK (Leaders In New Knowledge) Student, Department of Biology University of South Florida, undergraduate student
2003 Marina Si Chen, Summer Research Volunteer, King High School, high school student
2003 Sydnor M. Richkind, Summer Research Volunteer, Hillsborough High School, high school student (currently a graduate student in University of Florida)
2004-present Kristin Landis, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab
2004 Marina Si Chen, Summer Research Hourly Technician, Emory University, undergraduate student
2005-present Vesna Minic, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab
2005- Joan McCallum, Cancer Biology Program, Wayne State University, one lab rotation in my lab
2006-present Mike Frezza, Cancer Biology Program, Wayne State University, one lab rotation and Ph.D. student in my lab
2006 Benchamart Moolmuang, Cancer Biology Program, Wayne State University, one lab rotation in my lab
2006 Andy Yang, Summer Research Student, Webster Thomas High School, New York
2006 Marina Si Chen, Summer Research Hourly Technician, Emory University, undergraduate student
2006 Justin Shaya, Summer Research Student, West Bloomfield High School, MI

Theses/ Dissertation or Comprehensive Examination Committees

Ph.D. Dissertation Committees

1995 Kirti G. Goyal, Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine (Advisor: Dr. Leaf Huang)
1997 Jie-Gen Jiang, Pathology, University of Pittsburgh School of Medicine (Advisor: Dr. Reza Zarnegar), graduated in 12/97
1997-1998 Marni Brisson, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. Leaf Huang)
1997-1998 Donald Schwartz, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. John Lazo)
1996-1998 Robert Rice, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. John Lazo)
1996-1999 Cheryl Fattman, Pharmacology, University of Pittsburgh School of Medicine (Advisor: Dr. Q. Ping Dou)
1999-2002 David M. Smith, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (graduated; Ph.D. Advisor: Dr. Q. Ping Dou)

2000-2004 Kenyon Daniel, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Graduated; Ph.D. Advisor: Dr. Q. Ping Dou)

2001-2005 Deborah Kuhn, Cancer Biology Ph.D. Program (IOP), University of South Florida College of Arts and Sciences (Advisor: Dr. Q. Ping Dou). Graduated in November, 2004.

2002-2003 Audrey Burns, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Q. Ping Dou)

2002-2003 Sam Falsetti, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Q. Ping Dou)

2003 Bonnie Goodwin, Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine (Advisor: Dr. Duane Eichler)

2004 Kristin Landis, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)

2005 Vesna Minic, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)

2006 Mike Frezza, Cancer Biology Program, Wayne State University (Advisor: Dr. Q. Ping Dou)

Comprehensive Examination Committees

1994 Xiang Gao, Pharmacology, University of Pittsburgh School of Medicine

1995 Jeff Phillips, Pharmacology, University of Pittsburgh School of Medicine

1995 Chialin Cheng, Pharmacology, University of Pittsburgh School of Medicine

1995 Cheryl Fattman, Pharmacology, University of Pittsburgh School of Medicine

1996 Marni Brisson, Pharmacology, University of Pittsburgh School of Medicine

2003 Deborah Kuhn, Cancer Biology Ph.D. Program, University of South Florida College of Arts and Sciences

Research Associates and Others

2006-present Carol Maconochie, Administrative Assistant (Supervisor: Dr. Q. P. Dou)

2006-present Zhiyong Yu, Ph.D., Post-Doctoral Fellow

2006-present Guoqing Shi, Ph.D., Visiting Scholar

2005-present Huanjie Yang, Ph.D., Post-Doctoral Fellow

2004-present Cindy (Qiuzhi) Cui, Technician

2003-present Di Chen, Ph.D., Research Associate

2005-2006 Jaiwei Ren, Technician

2004-2006 Marcianna Norris, Administrative Assistant (Supervisor: Dr. Q. Ping Dou)

2006 Nivedita Tiwari, M.S., Sr. Research Assistant

2006 Lihua Li, M.D., Visiting Scholar

2005-2006 Haiyan Pang, Ph.D., Research Associate

2005 Alejandro Diez, M.D., Physician Intern

2004-2005 Shirley Adanta Orlu, Research Assistant

2005-2005 Yezhou Sun, Student Assistant

2004-2005 Kenyon Daniel, Ph.D., Post-Doctoral Fellow

2003-2004 MaryAnn Sparkman, Administrative Assistant (Supervisor: Dr. Q. Ping Dou)

2003-2004 Mohammad Bhuiyan, Ph.D., Research Associate

2000-2003 Aslamuzzaman Kazi, Ph.D., Research Associate

2002 Robin Shear, Research Volunteer

2000-2002 Sherry Zhong, Research Assistant

2001-2002 Puja Gupta, Research Volunteer
2000-2001 Hongwei Wang, Research Assistant
2000-2001 Kenyon Daniel, Research Assistant
1998-2000 Sangkil Nam, Ph.D., Research Associate
2000 Gen Wang, Ph.D., Research Associate
1999-2000 Xiaoxia Zhang, M.S., Research Assistant
1998-2000 Gui Gao, Ph.D., Research Associate
1998-2000 Benyi Li, M.D., Research Associate
1998-1999 Roland Cooper, Ph.D., Research Associate
1998 Jieliu Tang, Ph.D., Research Associate
1994-1998 Bing An, Research Associate
1996-1998 Terence F. McGuire, Ph.D., Instructor
1997-1998 Yibing Peng, M.S., Research Assistant
1995-1996 Jia-Rui Jin, Visiting Scholar
1995 Leilei Zhang, Visiting Scholar

GRANT SUPPORT:

Completed support

American Cancer Society Institutional Research Grant. Cyclins, transcription and defective growth control in cancer. Principal Investigator: Qing Ping Dou. 10/01/93-06/30/95.

Agreement with Beth Israel Hospital. Molecular Biology of Aging. Principal Investigator: Jeanne Y. Wei. 1994.

NIH R01. Molecular Biology of G1/S Regulation in Murine Cells. 07/01/93-06/30/96. Subcontract (Principal Investigator: Arthur B. Pardee)

NIH Shannon Award. Functions of RB-protease(s) in apoptosis. Principal Investigator: Qing Ping Dou. 09/15/95-08/31/97 (replaced by R29 on 04/14/96).

UPCI Breast Cancer Pilot Grant. Induction of p53-independent apoptosis and treatment of human breast cancer. Principal Investigator: Qing Ping Dou. 03/15/96-09/30/97.

NIH R29. Functions of RB-protease(s) in apoptosis. Principal Investigator: Q. Ping Dou (50%). 04/15/96-02/28/01.

NIH R01. Growth Inhibition by IL-2 of IL2R+ oral carcinomas. Principal Investigator: Q. Ping Dou (10%). 04/01/98-03/31/01. (a subcontract from University of Pittsburgh)

Department of the Army Advanced Cancer Detection Center Research Grant (Moffitt). Significance of Bax-Dependent Apoptosis in Prevention and Detection of Human Prostate and Lung Cancer. Principal Investigator: Q. Ping Dou. 10/01/00-9/30/01.

Administrative Supplement from Moffitt Cancer c ontrol. Co-Principal Investigato r: Q. Ping Dou.

Administrative Supplement from Moffitt Foundation. Co-Principal Investig ator: Q. Ping Dou.

Agreement from University of North Taxes. Co-Principal Investigator: Q. Ping Dou.

NIH R03. Tea Targeting Proteasom e: A Role in Cancer Prevention. Pr incipal Investigator: Q. Ping Dou (10%). 07/01/01-06/30/03.

Supplement for Correlative Studies Related to es trogen Receptor Negative (ER-negative) Breast Cancer (Moffitt CCOP Research Base). PI: Krischer; Co-Investigator: Q. Ping Dou.

U10 CA81920. A Clinical Trial of the Action of Isoflavones in Breast Neoplasia: Adm inistration Prior to Mastectomy or Lumpectomy - A Pilot Study. PI: Krischer; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

U10 CA81920. The Specific Role of Isoflavones in re ducing Prostate Cancer Risks. PI: Krischer; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

U10 CA81920. A Random ized Pilot C linical Trial of the Action of Isoflavones and Lycopene in Localized Prostate Cancer: Adm inistration Prior to radic al Prostatectomy. PI: Krischer ; Co-Investigator: Q. Ping Dou. 12/01/01-11/30/03

Approved but not funded

American Cancer Society. Induction of an RB-a ssociated phosphatase and cancer cell apoptosis **(Score: the second decile).** Principal Investigator: Q. Ping Dou. 01/01/97-12/31/99.

American Institute for Cancer Research. Tea polyphenols target proteasom e-mediated Bax degradation pathway: Significance in pr ostate cancer prevention and treatment **(Score: 2.92).** Principal Investigator: Q. Ping Dou. 02/01/00- 01/31/03.

Present support

NIH R01. Roles of polymorphic COMT, tea polyphenols and proteasome in cancer prevention. 20% Effort (PI: Q. Ping Dou). 04/01/ 06-03/31/11.

DOD Breast Cancer Research Pr ogram/IDEA Award. Synthetic β-Lactam Antibiotics as A Selective Breast Cancer Cell Apoptosis Inducer: Significance in Breast Cancer Prevention and Treatment. 20% Effort (PI: Q. Ping Dou). 03/01/04-3/31/07.

NIH R01. N-Thiolated β-Lactams. Co-Principal Investigator: Q. Ping Dou (5%) (PI: Ed Turos). 03/01/02-02/28/07.

DOD Breast Cancer Research Program -Concept Award. Examination of potential anti-tumor activity of N-thiolated β-lactam antibiotics in nude mice bearing human breast tumors. 5% Effort (PI: Q. Ping Dou). 10/01/04-09/30/06.

NIH R03. The Proteasome as Molecular Target of Grape Polyphenols. 5% Effort (PI: Q. Ping Dou). 12/01/04-11/30/06.

Wayne State University President's Research Enhancement Program Proposal. Enhancing chemo- and photodynamic therapy in breast cancer using nanotechnology. (Co-I: Q. Ping Dou; PI: Jayanth Panyam). 06/01/06-05/31/08.

VA Merit. Ubiquitin Protease System in Malignant Pleural Mesothelioma. 10% Effort. (Co-I: Q. Ping Dou; PI: Anil Wali). 09/01/06-08/31/10.

NIH R21. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/07-06/30/09.
(**2.8 percentile**)

NIEHS P50 ES012395. Center for Urban African American Health. 3.0% Effort (Co-I: Q. Ping Dou; PI: John Flack). 06/01/05-05/31/07.

T32-CA09531-19 NIH Training Grant. "Training Program in the Biology of Cancer" (Ms. Kristin R. Landis-Piwowar; Mentor: Q. Ping Dou) 09/01/05-8/31/07.

NCI/NIH the Cancer Center Support Grant (PI: Ruckdeschel). Population Studies & Prevention Program (Program PI: Schwartz; Co-I: Q. Ping Dou, 5%) 10/01/05-9/31/10.

Karmanos Cancer Institute Startup funds. Principal Investigator: Q. Ping Dou. 08/01/03 -.

Karmanos Cancer Institute Indirect Account. Principal Investigator: Q. Ping Dou. 08/01/03 -.

Pending support

NIH R01. Elucidation of molecular mechanisms for the ubiquitin-proteasome pathway in malignant pleural mesothelioma (Co-I: Q. Ping Dou; PI : Anil Wali). 10/01/05-9/30/10. Total Direct Costs: $1,250,000;

NIH R21. Molecular Study on Novel NCI Potential Anti-tumor Drugs. 15% Effort (PI: Q. Ping Dou). 04/01/05-03/31/07.

NIH R03. Targeting tumor endogenous copper with clioquinol. 5% Effort (PI: Q. Ping Dou). 07/01/05-06/30/07.

NIH R21. Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.

Alliance For Cancer Gene Therap y. Dual-agent nanoparticles to overcom e drug resis tance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/08. T

DOD Breast Cancer Research Program -Concept Award. Determination of potential anti-cancer activity of synthetic acetylated EGCG analog pr odrugs in nude m ice bearing hum an breast tumor xenografts. 5% Effort (PI: Q. Ping Dou). 07/01/05-06/30/06.

DOD Breast Cancer Research Prog ram-Concept Award. Synchronized Gene Silencing and Drug Delivery to Overcome Drug Resistance in Breast Cancer. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/06.

NIH R01. Synthesis and evaluation of prodrugs of green tea polyphenol EGCG analogs. 20% Effort (PI: Q. Ping Dou). 12/01/05-11 /30/10.

DOD Prostate Cancer Research Program-Idea Development Award. MOLECULAR TARGETS OF NOVEL NCI POTENTIAL ANTICANCER DRUGS IN HUMAN PROSTATE CANCER CELLS. 20% Effort Total Direct Costs: $375,000; Total Indirect Costs: $191,250

The Michigan Technology Tri-Corridor Fund, Fiscal Year 2005 Co mpetition. DUAL-AGENT NANOPARTICLES TO OVERCOME DRUG RESISTANCE. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/05-06/30/07.

DOD Breast Cancer Research Program/IDEA Award. The potential use of the anti-alcoholism drug disulfiram in breast cancer preven tion and trea tment. 15% Effort (PI: Q. Ping Dou). 01/01/06-12/31/08.

DOD Prostate Cancer Research Program /Physician Research Training Award. Novel organic copper complex PDC-Cu for m olecular therapy of prostate cancer facilitated by PET imaging (PI: Fangyu Peng; Mentor: Q. Ping D ou). 10/01/05-09/30/10.

Susan Komen Foundation. Nanoparticle-mediated combination photodynamic and chemotherapy to overcome refractory tumors. 5% Effort (Co- PI: Q. Ping Dou; PI: J ayanth Panyam). 05/01/06-04/30/08.

Wilson Foundation. Targeting tum or endogenous coppe r with the antibiotic clioquinol: A novel approach for can cer-specific killing with no or low toxicity. 15% Ef fort (PI: Q. Ping Dou). 01/01/06-12/31/07.

VA Merit. Ubiquitin Protease System in Malig nant Pleural Mesothelioma. 10% Eff ort. (Co-I: Q. Ping Dou; PI: Anil Wali).

NIH R01. Copper as a novel target for determ ining fate of AR and prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 07/01/06-06 /30/11.

NIH R21 (resubmission). Dual-agent nanoparticles to overcome drug resistance. 5% Effort (Co-PI: Q. Ping Dou; PI: Jayanth Panyam). 07/01/06-06/30/08.

NIH R01. The Chinese Thunder of God Vine: Active Components & Biological Targets in Cancer. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/11.

NIH P01. AR-mediated cell cycle-dependent anti-apoptotic events in prostate cancer. (PI: Reddy GPV; Co-Directors: Dou QP and Menon M). Project #3: Targeting 26S proteasome for determining fate of AR and prostate cancer cells. 40% Effort (PI: Q. Ping Dou). 12/01/06-11/30/11.

DOD Breast Cancer Research Program -Concept Award. Chemosensitization of human breast cancer cells by an active compound purified from the Chinese medicine Thunder of God vine. 5% Effort (PI: Q. Ping Dou). 07/01/06-06/30/07.

NIH R01. Maspin in Hormone Refractory Prostate Cancer Intervention (Co-I: Q. Ping Dou, 5%; PI: Shijie Sheng). 12/01/06-11/30/11.

NIH R01 (resubmission). Elucidation of molecular mechanisms for the ubiquitin-proteasome pathway in malignant pleural mesothelioma (Co-I: Q. Ping Dou; PI: Anil Wali). 12/01/06-11/30/11.

MICHIGAN ECONOMIC DEVELOPMENT CORPORATION (MEDC). Development of natural pharmaceuticals to protect against low-intensity radiation exposure. 5% Effort (Co-PI: Q. Ping Dou; PI: Michael C Joiner). 10/01/06-09/30/09.

National Natural Science Foundation of China (NSFC). Synthesis and Mechanistic Study of Catechin Glycosides as Proteasome Inhibitors. Co-PI: Q. Ping Dou (PI: Sheng Biao Wan). 10/01/06-09/30/08.

DOD Prostate Cancer Research Program -Idea Development Award. Targeting the proteasome/NFκB/ Androgen receptor-mediated survival pathway to chemosensitize human prostate cancer cells. 20% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

DOD Breast Cancer Research Program/IDEA Award. Overcoming breast cancer drug resistance by a medicinal compound isolated from Indian Winter Cherry. 15% Effort (PI: Q. Ping Dou). 12/01/06-11/30/09.

Wayne State University Faculty Graduate Research Assistantship (GRA). Gold-Containing Compounds as Proteasome Inhibitors and Apoptosis Inducers and their Potential Use for Breast Cancer Treatment. 08/17/07-08/16/08.

Fund for Cancer Research. Targeting the proteasome/ NFκB/ Androgen receptor-mediated survival pathway to chemosensitize human prostate cancer cells. (PI: Q. Ping Dou). 02/01/07-01/31/08.

NIH R01. Chemoprevention of Malignant Mesothelioma (Co-I: Q. Ping Dou, 5%; PI: Anil Wali). 10/01/07-9/30/12.

The Harry A. and Margaret D. Towsley Foundation. Prevention Program Research Projects. (PI: Q. Ping Dou). 01/01/08-12/31/12.

National Natural Science Foundation of China (NSFC). Synthesis and structure-activity-relationship study of proteasome-targeted catechin analogs and their antitumor activities. Co-PI: Q. Ping Dou (PI: Sheng Biao Wan). 10/01/07-09/30/09.

EHS Center in Molecular and Cellular Toxicology with Human Applications at Wayne State University. Targeting the carcinogenic pollutant cadmium in human breast cancer cells. (PI: Q. Ping Dou). 05/01/07-04/30/08.

PUBLICATIONS:

Original Observations in Referred Journals

1. Chen KY and Dou QP. NAD$^+$ stimulated the spermidine-dependent hypusine formation on the 18,000-dalton protein in cytosolic lysates derived from NB-15 mouse neuroblastoma cells. FEBS Letters, 1988; 229: 325-328
2. Chen KY and Dou QP. Isolation and characterization of an 18,000-dalton hypusine-containing protein from NB-15 mouse neuroblastoma cells. Biochem. Biophys. Acts, 1988; 971: 21-28
3. Dou QP and Chen KY. Two hypusine-containing proteins identified by metabolic labeling in chick embryo fibroblasts. J. Chin. Chem. Soc., 1989; 36: 443-450
4. Dou QP and Chen KY. Characterization and reconstitution of a cell-free system for NAD$^+$-dependent deoxyhypusine formation on the 18 kDa elF-4D precursor. Biochem. Biophys. Acts, 1990; 1036: 128-137
5. Bradley DW, Dou QP, Fridovich-Keil JL and Pardee AB. Transformed and non-transformed cells differ in stability and cell cycle regulation of a binding activity to the thymidine kinase promoter. Proc. Natl. Acad. Sci. USA, 1990; 87: 9310-9314
6. Dou QP, Fridovich-Keil JL and Pardee AB. Inducible proteins binding to the murine thymidine kinase promoter in late G1/S phase. Proc. Natl. Acad. Sci. USA, 1991; 88: 1157-1161
7. Fridovich-Keil JL, Gudas JM, Dou QP, Bouvard I and Pardee AB. Growth-responsive expression from the murine thymidine kinase promoter: Genetic analysis of DNA sequences. Cell Growth. Diff., 1991; 2: 67-76
8. Dou QP, Markell PJ, and Pardee AB. Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and cdc2 kinase. Proc. Natl. Acad. Sci. USA, 1992; 89: 3256-3260
9. Dou QP, Levin AH, Zhao S, and Pardee AB. Cyclin E and cyclin A as candidates for the restriction point protein. Cancer Res. (*Advances in Brief*), 1993; 53: 1493-1497
10. Dou QP, Zhao S, Levin AH, Wang J, Helin K, and Pardee AB. G1/S-regulated E2F-containing complexes bind to the mouse thymidine kinase gene promoter. J. Biol. Chem., 1994; 269: 1306-1313

11. Dou QP, Molnar G, and Pardee AB. Cyclin D1/cdk2 kinase is present in a G1 phase-specific protein complex Yi1 that binds to the mouse thymidine kinase gene promoter. Biochem. Biophys. Res. Commun., 1994; 205: 1859-1868
12. Dou QP, An B and Will PL. Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. Proc. Natl. Acad. Sci. USA, 1995; 92: 9019-9023
13. Dou QP, An B and Yu C. Activation of cyclin E-dependent kinase by DNA-damaging signals during apoptosis. Biochem. Biophys. Res. Commun., 1995; 214: 771-780
14. Dou QP and Lui VWY. Failure to dephosphorylate retinoblastoma protein in drug resistant cells. Cancer Res. (*Advances in Brief*), 1995; 55: 5222-5225
15. An B and Dou QP. Cleavage of retinoblastoma protein during apoptosis: an interleukin 1 β-converting enzyme-like protease as candidate. Cancer Res. (*Advances in Brief*), 1996; 56: 438-442
16. Dou QP, Pardee AB and Keyomarsi K. Cyclin E—a better prognostic marker for breast cancer than cyclin D? Nature Medicine, 1996; 2: 254
17. An B, Jin J-R, Lin P and Dou QP. Failure to activate interleukin 1β-converting enzyme-like proteases and to cleave retinoblastoma protein in drug-resistant cells. FEBS Letters; 1996; 399: 158-162
18. Dou QP, An B, Antoku K and Johnson DE. Fas stimulation induces RB dephosphorylation and proteolysis that is blocked by inhibitors of the ICE protease family. J. Cell. Biochem., 1997; 64: 586-594
19. Molnar GM, Crozat A, Kraeft S-K, Dou QP, Chen LB and Pardee AB. Association of the mammalian helicase MAH with the pre-mRNA splicing complex. Proc. Natl. Acad. Sci. USA, 1997; 94: 7831-7836
20. Fattman CL, An B and Dou QP. Characterization of interior cleavage of retinoblastoma protein in apoptosis. J. Cell. Biochem. (A figure was selected as the cover of the journal), 1997; 67: 399-408
21. An B, Dineley KE, Zhang LL, Termin TA, Meijer L and Dou QP. Involvement of RB kinases and phosphatases in life and death decisions. Oncology Reports, 1997; 4: 1129-1134
22. An B, Johnson DE, Jin J-R, Antoku K and Dou QP. Bcl-2- and CrmA-inhibitable dephosphorylation and cleavage of retinoblastoma protein during etoposide-induced apoptosis. Intl. J. Mol. Med., 1998; 1: 131-136
23. Fattman CL, An B, Sussman L and Dou QP. p53-independent dephosphorylation and cleavage of retinoblastoma protein during tamoxifen-induced apoptosis in human breast carcinoma cells. Cancer Lett., 1998; 130: 103-113
24. An B, Goldfarb RH, Siman R and Dou QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death & Diff., 1998; 5: 1062-1075
25. Dou QP, McGuire TF, Peng Y and An B. Proteasome inhibition leads to significant reduction of Bcr-Abl expression and subsequent induction of apoptosis in K562 human chronic myelogenous leukemia cells. J. Pharm. Exp. Ther., 1999; 289: 781-790
26. Reichert TE, Nagashima S, Kashii Y, Stanson J, Gao G, Dou QP and Whiteside TL. Interleukin-2 expression in human carcinoma cell lines and its role in cell cycle progression. Oncogene, 2000; 19: 514-525
27. Li B and Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. Proc. Natl. Acad. Sci. USA, 2000; 97: 3850-3855
28. Gao G and Dou QP. G1 phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. Mol. Pharm., 2000; 58: 1001-1010

29. Gao G and Dou QP. N-Terminal Cleavage of Bax Protein by a Mitochondrial Calpain Activity Generates a Potent Proapoptotic 18 KD Fragment That Induces Bcl-2-Independent Cytochrome C Release and Apoptotic Cell Death. J Cell. Biochem., 2000; 80: 53-72
30. Sun J, Nam S, Lee C-S, Li B, Coppola D, Hamilton AD, Dou QP (co-corresponding author) and Sebti SM. CEP1612, a dipeptidyl proteasome inhibitor, induces $p21^{WAF1}$ and $p27^{KIP1}$ expression and apoptosis and inhibits the growth of the human lung adenocarcinoma A-549 in nude mice. Cancer Res. (*Advances in Brief*), 2001; 61: 1280-1284
31. Fattman CL, Delach S, Dou QP and Johnson DE. Sequential two-step cleavage of the retinoblastoma protein by caspase -3 during etoposide-induced apoptosis. Oncogene, 2001; 20: 2918-26
32. Nam S, Smith DM and Dou QP. Inhibition of proteasome activity *in vitro* and *in vivo* by ester bond-containing tea polyphenols. J. Biol. Chem., 2001; 276: 13322-13330
33. Nam S, Smith DM and Dou QP. Tannic acid potently inhibits tumor cell proteasome activity, increases p27 and Bax expression, and induces G_1 arrest and apoptosis. Cancer Epidemiol Biomarkars Prev, 2001; 10: 1083-8
34. Smith DM and Dou QP. Green tea polyphenol epigallocatechin inhibits DNA replication and consequently induces leukemia cell apoptosis. Intl. J. Mol. Med, 2001; 7: 645-652
35. Smith DM, Kazi A, Smith L, Long ET, Turos E and Dou QP. A Novel β-Lactam Antibiotic Activates Tumor Cell Apoptotic Program by Inducing DNA Damage. Mol Pharm. 2002; 61: 1348-1358
36. Kazi A, Smith DM, Zhong Q and Dou QP. Inhibition of Bcl-X_L phosphorylation by tea polyphenols is associated with prostate cancer cell apoptosis. Mol Pharm (Accelerated Communication) 2002; 62: 765-771
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ABSTRACTS FOR POSTER PRESENTATION IN THE LAST FIVE YEARS:

Fattman CL and Dou QP. Distinct ICE-like proteases mediate cleavage of retinoblastoma protein and poly(ADP-ribose) polymerase during apoptosis. AACR 88th annual Meeting, San Diego, California, April 12-16, 1997.

Dou QP, An B and Fattman CL. Retinoblastoma protein and the regulation of apoptosis. 7th SCBA International Symposium, Toronto, Canada, July 6-11, 1997.

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Fattman CL, An B, Zhang LL, Dineley K and Dou QP. Dephosphorylation and cleavage of the retinoblastoma protein during p53-dependent and -independent apoptosis of human breast carcinoma cells. Department of Defense, U.S. Medical Research and Material Command, Breast Cancer Research Program: An Era of Hope, Washington, D.C., October 31-November 4, 1997.

Dou QP. Cell cycle checkpoint proteins as apoptosis therapeutic targets. NATO Advanced Research Workshop at H. Lee Moffitt Cancer Center and Research Institute University of South Florida, Protein-Protein and Protein-Lipid Interactions in Signal Transduction: Use of Small Synthetic Molecules as probes and Therapeutic Agents, Clearwater Beach, Florida, December 5-9, 1997

Johnson DE, Rabinovitz A, Dou QP, Delach SM and Fattman CL. VP-16 treatment of T-leukemic cells results in activation of Cathepsins D and L via a caspase-dependent pathway. 1999 Annual Meeting of the American Society of Hematology, New Orland, Louisiana, December 2-7, 1999

Dou QP and Li B. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. AACR 91st Annual Meeting, San Francisco, CA, April 1-5, 2000

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Gao G and Dou QP. G1 phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. 29th Annual Scientific Meeting of the International Society for Experimental Hematology, Tampa, FL, July 8-11, 2000

Smith DM and Dou QP. Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000

Smith DM, Nam S and Dou QP. Studies on tumor related targets of green tea polyphenols. Poster presentation. 2nd International Conference. Mechanisms of Cell Death and Disease: Advances in Therapeutic Intervention, North Falmouth, MA, June 2-6, 2001

Daniel KG, Zhong Q, Gupta P and Dou QP. Etoposide induces activation of calpain in early stages of apoptosis. Poster presentation. 22nd Annual South East Pharmacology Society Meeting (SEPS), Drug Development Symposium (from Bench to Bedside), Clearwater, FL, October 4-6, 2001

Kazi A, Smith DM, Zhong Q and Dou QP. Down Regulation of Bcl-X_L Protein Expression by Green Tea Polyphenols Is Associated with Prostate Cancer Cell Apoptosis. Poster presentation. AACR-NCI-EORTC. Molecular Targets and Cancer therapeutics, Miami Beach, FL, October 29-November 2, 2001

Dou QP, Kazi A, Smith DM and Kuhn DJ. Tea Polyphenols Target Proteasome-Mediated Bax Degradation in Prostate Cancer Cells: A Potential Role in Cancer Prevention. Poster presentation. An AACR Special Conference in Cancer Research. New Discoveries in Prostate Cancer Biology and Treatment, Naples, FL, December 5-9, 2001

Nam S, Dalton WS, Trotti AM, Dou QP and Calvin DP. Cell adhesion to fibronectin (FN) through β1 integrins results in cell adhesion mediated ionizing radiation resistance (CAM-RR) in human LNCaP prostate cancer cells: the potential involvement of proteasome chymotrypsin-like activity. Poster presentation. AACR 93rd Annual Meeting, San Francisco, CA, April 6-10, 2002

Kazi A, Hill R, Long TE, Turos E, Dou QP. Selective induction of apoptosis in human tumor cells by novel N-thiolated beta-lactams. Poster presentation. Molecular Targets for Cancer Therapy, St. Pete Beach, FL, October 11-15, 2002

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David M. Smith, Zhigang Wang, Aslamuzzaman Kazi, Kenyon G. Daniel, Lian-hai Li, Tak-Hang Chan, and Q. Ping Dou. Green tea polyphenol proteasome inhibitors as potential cancer-preventative agents: computational design, organic synthesis and biological evaluation. Poster presentation. AACR Special Conference, Proteases, Extracellular Matrix, and Cancer, Hilton Head Island, South Carolina, October 9-13, 2002

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Kazi A, Hill R, Long TE, Turos E, and Dou QP. Selective Induction of Apoptosis in Human Tumor Cells by Novel N-thiolated Beta-Lactams. Poster presentation. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003

Smith DM, Wang Z, Kazi A, Daniel KG Li LH, Chan TH and Dou QP. Green tea polyphenol proteasome inhibitors as potential cancer-preventative agents: computational design, organic, synthesis and biological evaluation. Poster presentation. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of β-Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, Detroit, MI, September 8, 2004

Di Chen, Marina Chen, Kenyon G. Daniel, Deborah J. Kuhn, Kristin Landis, and Q. Ping Dou. Dietary Flavonoids as Proteasome Inhibitors and Apoptosis Inducers in Human Leukemia Cells and Their Structure-activity Relationships. Poster presentation. Third Annual AACR Cancer Prevention Meeting, Seattle, Washington, October 16-20, 2004

Deborah Kuhn, Yang Wang, Vesna Minic, Cristina Coates, G. Suresh Kumar Reddy, Kenyon, G. Daniel, Jeung-Yeop Shim, Di Chen, Kristin R. Landis-Piwowar, Edward Turos, And Q. Ping

Dou, Ph.D. Relationships of structures of N-methylthiolated beta-lactam antibiotics to their apoptosis-inducing activity in human breast cancer cells. Poster presentation. ERA OF HOPE, Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania, June 8-11, 2005

Minic V, Kuhn D, Daniel K, Chen D, Landis K, Turos E, Dou QP. Potential Use of β-Lactam antibiotics in Cancer Prevention and Therapy. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Di Chen, Marina Chen, Kenyon G. Daniel, Deborah J. Kuhn, Kristin Landis, and Q. Ping Dou. Dietary Flavonoids as Proteasome Inhibitors and Apoptosis Inducers in Human Leukemia Cells and Their Structure-activity Relationships. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Kristin R. Landis-Piwowar, Deborah J. Kuhn, Sheng Biao Wan, Di Chen, Tak Hang Chan, and Q. Ping Dou. Evaluation of Proteasome-Inhibitory and Apoptosis-inducing Potencies of Novel (-)-EGCG Analogs and their Prodrugs. Poster presentation. Graduate student recruitment for Cancer Biology Program, Karmanos Cancer Institute and Wayne State University, April 2, 2005

Di Chen, Kenyon G. Daniel, Marina S. Chen, Deborah J. Kuhn, Kristin R. Landis Piwowar, Wai Har Lam, Larry M. C. Chow, Tak Hang Chan and Q. Ping Dou. Dietary and synthetic polyphenols as proteasome inhibitors and apoptosis inducers in human cancer cells. 5th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, Wayne State University School of Medicine, Detroit, MI 48201, April 23, 2005

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang, Fazlul H. Sarkar, G. Prem Veer Reddy, Shijie Sheng, Raul A Barrea and Q. Ping Dou. Clioquinol, A Therapeutic Agent For Alzheimer's Disease, Has Proteasome-Inhibitory, Apoptosis-Inducing And Anti-Tumor Activities In Prostate Cancer Cells And Xenografts. 6th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Huanjie Yang, Di Chen, Qiuzhi Cindy Cui, Xiao Yuan, and Q. Ping Dou. Celastrol, A Triterpene Extracted From The Chinese Thunder Of God Vine, Is A Potent Proteasome Inhibitor And Suppresses Human Prostate Cancer Growth In Nude Mice. 6th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006 (The First Place Poster Award)

Xiaohua Li, Di Chen, Shuping Yin, Yiwei Li, Huanjie Yang, Kristin R. Landis-Piwowar, Fazlul Sarkar, Prem Veer G. Reddy, Q. Ping Dou, Shijie Sheng. Proteasome Inhibition Up-regulates Apoptosis-sensitizing Maspin. 6th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang and Q. Ping Dou. Disulfiram, a clinically used anti-alcoholism drug and copper-binding agent, induces apoptotic cell death in breast cancer cultures and xenografts *via* inhibition of the proteasome activity. The AACR International Conference on Frontiers in Cancer Prevention Research, Boston, MA, November 12 - 15, 2006.

Kristin R. Landis-Piwowar, Deborah J. Kuhn, Sheng Biao Wan, Di Chen, Tak Hang Chan, and Q. Ping Dou. Evaluation of Proteasome-Inhibitory and Apoptosis-inducing Potencies of Novel (-)-EGCG Analogs and their Prodrugs. Poster presentation. Karmanos Cancer Institute the Protease and Cancer Program Annual Retreat, Scott Hall, Wayne State University, Detroit, MI, December 1, 2006

Landis-Piwowar KR, Chen D, Chen M, Cui QZ, Dou QP. The dietary flavonoid apigenin inhibits proteasome activity and suppresses breast cancer growth in nude mice. Poster Presentation. In the Forefront of Basic and Thanslational Cancer Research, AACR/JCA 7th Joint Conference, Waikoloa, HI, January 21 - 25, 2007.

Khdair A, Chen D, Dou QP, Panyam J. Nanoparticle-mediated Chemo- and Photodynamic Therapy for Drug Resistant Tumors. Poster Presentation. 10th Annual Meeting of Regional Cancer Center Consortium for the Biological Therapy of Cancer, International Hotel and MBNA Conference Center, Cleveland, Ohio, February 8-9, 2007.

Di Chen, Qiuzhi Cindy Cui, Huanjie Yang, Raul A Barrea, Fazlul H. Sarkar, Shijie Sheng, Bing Yan, G. Prem Veer Reddy, and Q. Ping Dou. Clioquinol, a therapeutic agent for Alzheimer's disease, has proteasome-inhibitory, androgen receptor-suppressing, apoptosis-inducing, anti-angiogenic and anti-tumor activities in human prostate cancer cells and xenografts. The 2007 AACR Annual Meeting, Los Angeles, CA, April 14 - 18, 2007.

INVITED ORAL PRESENTATIONS (IN THE LAST FIVE YEARS):

Dou QP. Apoptosis control and cancer. Department of Pharmacology, University of Pittsburgh School of Medicine, February 7, 1997

Dou QP. Apoptosis control and cancer. *Cephalon*, Inc., March 11, 1997

Dou QP. RB and apoptosis. University of Pittsburgh Cancer Institute, Molecular Oncology Seminar Series, April 16, 1997

Dou QP. Activation of apoptotic death program in human cancer. H. Lee Moffitt Cancer Center & Research Institute at the University of South Florida, April 28, 1997

Dou QP. Invited Speaker. Retinoblastoma protein and the regulation of apoptosis. 7th SCBA International Symposium, Toronto, Canada, July 6-11, 1997.

Dou QP. Cell cycle and Apoptosis. University of Pittsburgh Cancer Institute, FAS-L Club, August 12, 1997.

Dou QP. Apoptosis regulation in breast cancer. Second Annual Pittsburgh Minisymposium on Basic and Translational Research in Breast Cancer, Center for Environmental and Occupational Health and Toxicology, University of Pittsburgh, August 16, 1997

Dou QP. Invited speaker. RB and apoptosis control. Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, September 25, 1997

Dou QP. Invited Speaker. Targeting the Apoptotic Signaling Pathway in Human Cancer. Departments of Biochemistry & Molecular Biology and Microbiology & Immunology, University of North Texas Health Science Center at Fort Worth, September 29, 1997

Dou QP. Invited Speaker and Session Chairman. Putative roles of retinoblastoma protein in apoptosis. 2nd World Congress on Advances in Oncology, Athens, Greece, 16-18 October, 1997

Dou QP. Invited Speaker. Cell cycle checkpoint proteins as apoptosis therapeutic targets. NATO Advanced Research Workshop at H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Protein-Protein and Protein-Lipid Interactions in Signal Transduction: Use of Small Synthetic Molecules as probes and Therapeutic Agents, Clearwater Beach, Florida, December 5-9, 1997

Dou QP. Targeting ubiquitin/proteasom-mediated protein degradation pathway in human cancers. Research Progress Seminar Series at H. Lee Moffitt Cancer Center and Research Institute and University of South Florida, Tampa, Florida, October 29, 1998

Dou QP. Invited Speaker. Targeting ubiquitin/proteasom-mediated protein degradation pathway in human cancers. Department of Pharmacology and Therapeutics, University of South Florida College of Medicine, Tampa, Florida, February 17, 1999

Dou QP. Invited Speaker. Bax degradation by the proteasome: a survival mechanism used by human cancer cells. Department of Biochemistry & Molecular Biology, University of South Florida College of Medicine, Tampa, Florida, October 15, 1999

Gao G and Dou QP. G1 phase-dependent Bcl-2 expression correlates with chemoresistance of human cancer cells. Oral presentation. 29th Annual Scientific Meeting of the International Society for Experimental Hematology, Tampa, FL, July 8-11, 2000

Smith DM and Dou QP. Green tea targets human tumor cell DNA synthesis and consequently induces apoptosis. Poster presentation. New Molecular Targets for Cancer Therapy, St. Petersburg Beach, FL, October 14-17, 2000

Dou QP. Invited Speaker. Proteasome inhibitors as novel anticancer drugs. Cancer Research and Biotechnology in the I-4 Corridor, Moffitt Cancer Center & Research Institute, Tampa, Florida, August 21, 2000

Dou QP. Invited Speaker. Therapeutic potential of proteasome inhibitors in cancer prevention and treatment. Moffitt Cancer Center Research Retreat, Saddlebrook Resort, FL, May 19, 2001

Smith DM and Dou QP. Drug Discovery: Hunting for Cancer-Specific Molecular Targets - from Natural to Synthetic Compounds. Drug Discovery and Developmental Therapeutics Seminar, Moffitt Cancer Center & Research Institute, June 21, 2001

Dou QP. Invited Speaker. Proteasome inhibitors. New drugs in hematologic malignancies, Institute Of Hematology and Medical Oncology, “Seragnoli”, University of Bologna, Bologna, Italy, November 12-14, 2001

Dou QP. Invited Speaker. Proteasome: a novel target for cancer prevention and treatment as well as anti-angiogenic therapy. Moffitt Grand Rounds, Moffitt Cancer Center & Research Institute, Tampa, FL, November 15, 2002

Dou QP. Invited Speaker. Identification of A Novel Molecular target for Anti-Copper and Anti-Angiogenic Therapies. Attenuon, L.L.C., San Diego, CA, November 25, 2002

Dou QP. Invited Speaker. Natural Proteasome Inhibitors and Chemoprevention. Karmanos Cancer Institute at Wayne State University, Detroit, MI, February 6, 2003

Dou QP. Invited Speaker. Proteasome, a novel target for anti-cancer drug discovery. Moffitt Scientific Retreat, Tampa, FL, February 15, 2003

Dou QP. Invited Speaker. Proteasome Inhibitors. Sopherion Therapeutics, Inc., New Haven, CT, March 13, 2003

Dou QP. Invited Speaker. TBN. Department of Pathology, Wayne State University, Detroit, MI, June 25, 2003 (rescheduled)

Daniel KG and Dou QP. Organic-copper complexes as a new class of proteasome inhibitors: the potential of converting a pro-angiogenic factor to a cancer cell-specific killer. Drug Discovery and Developmental Therapeutics Seminar, Moffitt Cancer Center & Research Institute, May 29, 2003

Dou QP. Invited Speaker. Chemoprevention: targeting the proteasome. Karmanos Scientific Retreat, Detroit, MI, August 22, 2003

Dou QP. Invited Speaker. The proteasome: a novel molecular target for cancer prevention and treatment. The Protease Group, Karmanos Cancer Institute, Detroit, MI, September 2, 2003

Dou QP. Invited Speaker. Proteasome inhibitors and chemoprevention. Great lakes chemoprevention retreat, Maumee Bay Resort, Ohio, September 13, 2003

Dou QP. Invited Speaker. Prostate Cancer Research Summary. Henry Ford Health System /WSU Prostate Journal Club, Detroit, MI, November 12, 2003

Dou QP. Invited Speaker. Green tea and cancer prevention. Presentation to Cancer Biology Candidate Students, Karmanos Cancer Institute, Detroit, MI, April 3, 2004

Dou QP. Invited Speaker. Synthetic beta-lactam antibiotics and a selective breast cancer cell apoptosis inducer: Significance in breast cancer prevention and treatment. The Breast Cancer Group, Karmanos Cancer Institute, Detroit, MI, May 6, 2004

Dou QP. Invited Speaker. Tea Polyphenols. Karmanos Cancer Institute Research Retreat, Detroit, MI, August 27, 2004

Dou QP. Invited Speaker. Discovery of Novel Small Molecules: Rational Design, Structure-Activity Relationships, Cellular Targets, and Potential Uses for Cancer Treatment and Prevention. The Developmental Therapeutics Group, Karmanos Cancer Institute, Detroit, MI, September 8, 2004

Dou QP. Invited Speaker. Proteasome Inhibitors: Killing *via* Tumor-Specific Signaling. Basic and Translational Aspects of Cancer Cell Signaling Research Retreat, Karmanos Cancer Institute, Detroit, MI, January 14, 2005

Dou QP. Invited Speaker. Searching for Natural and Pharmacological Proteasome Inhibitors for Cancer Prevention and Treatment. Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, April 13, 2005

Dou QP. Invited Speaker. Searching for Novel Polyphneol Proteasome Inhibitors for Cancer Prevention and Treatment. Department of Urology at the University of California San Francisco and San Francisco VA Medical Center, San Francisco, CA, April 28, 2005

Dou QP. Invited Speaker. Roles of polymorphic catechol-*O*-methyltransferase gene, tea polyphenols and proteasome in cancer prevention. Population Studies and Prevention Joint Meeting, Karmanos Cancer Institute, Detroit, MI, June 14, 2005

Dou QP. Invited Speaker. Tea polyphenols, Proteasome and Polymorphic Catechol-*O*-Methyltransferase: Use in Cancer Molecular Diagnosis, Prevention and Treatment. Department of Chemistry at McGill University and American Diagnostica Inc., Montreal, Quebec, Canada, August 22, 2005

Kristin Landis (Mentor: Dou QP). Selected Speaker. Molecular Studies for the Regulation of Green Tea Polyphenol Biological Function by the Polymorphic Gene Product Catechol-O-Methyltransferase. Wayne State University Graduate Student Research Day, Detroit, MI, September 22, 2005

Dou QP. Invited Speaker. Copper as a novel target for determining fate of AR and prostate cancer cells. Karmanos Cancer Institute Research Retreat, Detroit, MI, October 7, 2005

Dou QP. Searching for natural proteasome inhibitors for cancer prevention and anti-cancer drug discovery**.** Department of Pathology Retreat, Wayne State University School of Medicine, Detroit, MI, August 27, 2005 (canceled)

Landis-Piwowar KR, Wan SB, Daniel KG, Chen D, Chan TH, and Q. Ping Dou. Computational Modeling and Biological Evaluation of Methylated (-)-EGCG Analogs. Selected as a Minisymposium presentation. The 2006 AACR 97th Annual Meeting, Washington DC, April 1-5, 2006

Dou QP. Invited Speaker. Roles of Diet, Biometals, and Environmental Factors in Cancer Prevention. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China, April 19-20, 2006

Dou QP. Invited Speaker. Nutrient-Gene-Environment Interactions and Molecular Prevention of Cancer. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 24, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Therapies : - *From Nature to Laboratories and … back*. The Institution of Environmental Sciences, Shandong University, Jinan, Shandong, China, April 25, 2006

Dou QP. Invited Speaker. A Common Target of Dietary Factors, Traditional Medicine and Chemopreventive Agents in Human Prostate Cancer: the Significance in Molecular Prevention. 6th Annual Symposium, Michigan Prostate Research Colloquium, Basic and Clinical Advances in Prostate Cancer Research, University of Michigan Medical School, Towsley Center, Dow Auditorium, Ann Arbor, MI 48109, May 6, 2006

Dou QP. Invited Speaker. Molecular Prevention of Human Cancer: An Example of Diet-Gene-Environment Interaction. Institution of Environmental Health Sciences, Wayne State University, Detroit, MI, June 15, 2006.

Dou QP. Invited Speaker. A Lesson Learned from Thymidine Kinase Transcription at G1/S and later Stories. Symposium Honoring Dr. Pardee on the occasion of his 85th birthday. Boston, MA, June 24, 2006.

Dou QP. Invited Speaker. Discovery of Novel Natural and Synthetic Compounds for Molecular Prevention of Human Cancer. Henry Ford Health Systems, Detroit, MI, August 3, 2006.

Dou QP. Invited Speaker. Green Tea and Cancer Prevention. The Day of Wellness, Grosse Pointe War Memorial, Grosse Pointe, MI, September 16, 2006.

Kristin Landis (Mentor: Dou QP). A Novel Pro-drug of Green Tea Catechin (-)-EGCG as a Potential Anti-Breast Cancer Agent. Wayne State University Graduate Student Research Day, Detroit, MI, September 21, 2006. (Session Winner)

Dou QP. Invited Speaker. Green Tea and its Role in Cancer Prevention. Dow and Towsley Foundation Visit, Karmanos Cancer Institute Research, Detroit, MI, October 11, 2006

Dou QP. Invited Speaker. Molecular Cancer Prevention and Therapies. Shandong Institute of Cancer Prevention and Treatment, Jinan, Shandong, China, October 18, 2006

Dou QP. Invited Speaker. Discovery of Novel Small Molecules for Cancer Prevention and Therapies. Wuhan Botanical Garden/ Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, China, October 20, 2006

Dou QP. Invited Speaker. The Proteasome as a Potential Cellular Target of Organic Toxic Metals. The 3rd International Symposium on Persistent Toxic Substances, Beijing, China, October 22-25, 2006

Dou QP. Invited Speaker. Proteasome inhibitors for cancer prevention and treatment. Karmanos Cancer Institute the Protease and Cancer Program Annual Retreat, Scott Hall, Wayne State University, Detroit, MI, December 1, 2006

Dou QP. Invited Speaker. Converting the proangiogenic copper to a specific death inducer: significance in breast cancer prevention and treatment. Karmanos Cancer Institute the Breast Cancer Program Annual Retreat, HWRC 2nd Floor auditorium, Karmanos Cancer Institute, Wayne State University, Detroit, MI, March 2nd, 2007

Dou QP. Invited Speaker. Disulfiram. Molecular Therapeutics of Cancer, Gordon Research Conference, Colby-Sawyer College, New London, New Hampshire, July 22-27, 2007.

Dou QP. Invited Speaker. Green Tea Polyphenols as a Natural Tumor Cell Proteasome Inhibitor. The 3rd International Symposium on Functional Foods, PLANT POLYPHENOS, "*What would life be without plant polyphenols*?" Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, P.R. China, April 03-05 2008

Dou QP. Invited Speaker. TBN. The International Conference on Nutrition and Cancer, Elazig, Turkey, May 20-23, 2008